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Final Technical Report DARPA Award HR0011-08-1-0087 Drug Discovery, Design and Delivery

PIs: Burt Anderson, Richard Heller, Ed Turos, Mark Mclaughlin

Funding Period: August 22, 2008- February 21-2010 NCE to August 21, 2010

This was a joint project with four investigators. Three are located at the University of South Florida and one at Old Dominion University. The project aims to develop novel strategies to treat or prevent infectious caused by bacteria which pose as biothreat agents (*Bacillus anthracis*) or are recognized causes of emerging disease (*Bartonella* species).

The broad project-wide aims are as follows:

- Demonstrate potential novel compounds to effectively target and treat intracellular pathogens
  - Novel antibiotics (Turos/Anderson)
  - Anti-gene peptide nucleic acids (Mclaughlin/Anderson)
- Develop vaccine approach that can be utilized against biothreat/emerging agents of infectious diseases
  - Electroporation enhanced delivery (Heller)
  - Bacterial secretion systems as potential delivery mechanism (Anderson)

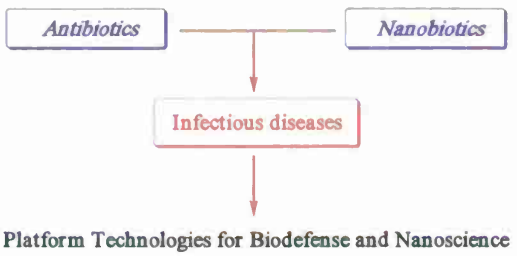
Progress toward the aims/goals of each subproject is outlined in the four reports which follow. Each report was prepared by the individual PI.

Final Report

Turos Subproject

Project Goals: To develop small molecule growth inhibitors and organic nanoparticles that can effect the growth of *Bartonella* bacteria

Research in our laboratory centers on the development of new antibiotic substances and novel nanoparticle-based biomaterials for the treatment and prevention of infectious diseases. More specifically, the aim of this DARPA investigation was to investigate the discovery of antibiotics and nanoparticle-based approaches for targeting and neutralizing the pathogenic growth of *Bartonella* bacteria. We thus divided the studies into two parts: (1) antibiotics and (2) nanoparticles.



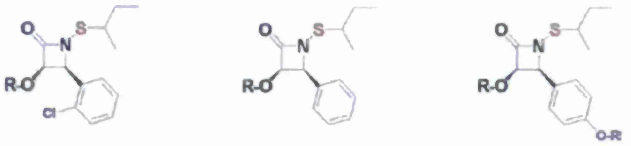
Part I. Discovery of small molecule anti-Bartonella antibiotics.

Sub-aim 1: Synthesize and characterize new N-thiolated beta-lactam antibiotics for biological testing against B. henselae **(collaborator: Anderson)**

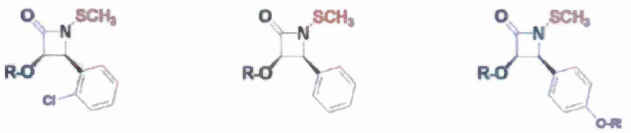
- *prepare and characterize new chemical derivatives of N-thiolated beta-lactams having lipophilic versus water-solubilizing side chains*
- *assay these new beta-lactam derivatives for in vitro bioactivity against B. henselae by determining minimum inhibitory concentration (in Anderson lab)*
- *reiterate synthesis-testing cycle to identify most potent beta-lactam variant against B. henselae*

Prior studies in our lab have shown that N-thiolated beta-lactams (synthesized within our lab) inhibit the growth of select pathogenic bacteria such as *Staphylococcus* and *Bacillus* in a manner that does not alter the natural microflora of community microbes. The compounds were found to have a unique mode of action in which microbial fatty acid biosynthesis is blocked but only in bacterial having high cytosolic levels of coenzyme A (CoA). However, for *Bartonella*, it has not been determined yet what the levels of CoA are in the cytosol, or more importantly, glutathione (which reduces antibacterial capabilities of the lactams, presumably by dethiolation of the lactam thiol side chain). Thus, we decided to examine the bioactivity of some of the compounds prevoiously studied ofr other microbes against *Bartonella* bacteria. These are shown below, and include two parallel series of N-thiolated derivatives: (1) sec-butylthio lactams, and (2) methylthio derivatives.

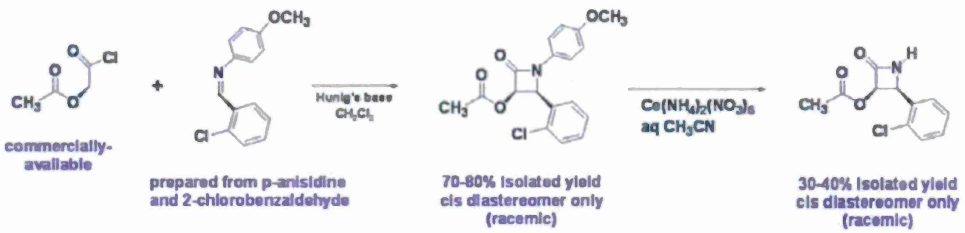
N-sec-Butylthio series

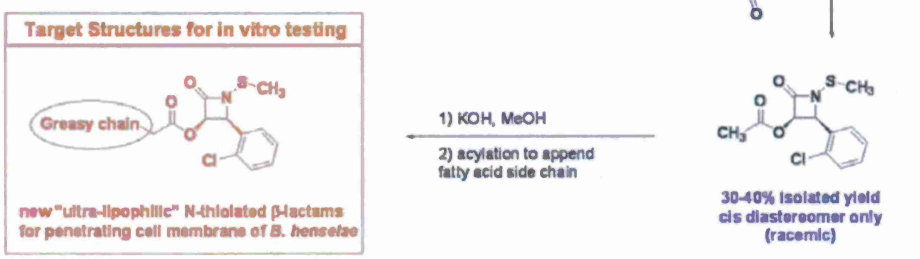


N-Methylthio series



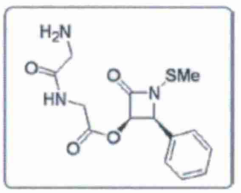
We also had interest in prepared lactam analogues carrying lipophilic acyl side chains at carbon-3 of the lactam ring, to examine if there might be a correlation between the length or branching of acyl residues versus bioactivity. Our aim was to try to "bust through" the cell membrane of *Bartonella* through attachment of highly lipid soluble residues at non-essential positions on the antibiotic. These 3-acyl analogues were prepared in our laboratory in four steps, as illustrated in the following scheme, with a variety of acyl moieties ranging in length from one to 10 carbons (straight chains).

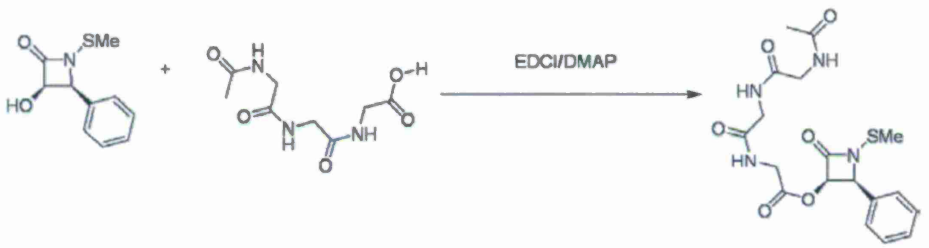




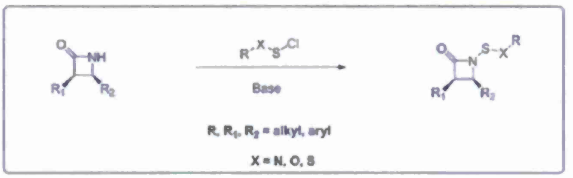
Along with these, we thought it would be insightful to prepare analogues having amino acid residues at carbon-3 of the lactam to determine if these compounds could have improved water solubility while retaining bioactivity. An example is shown below (in the box) along with a method we developed for attaching amino acid side chains directly onto an existing hydroxyl group at carbon-3 on the beta-lactam ring. We were successful in preparing N-protected amino acids beta-lactam derivatives (N-acetyl and N-t-BOC glycinyl, for instance), but we could not find conditions to remove these acyl

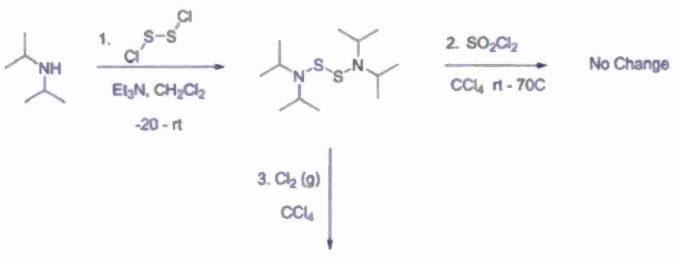
protecting groups from the side chain nitrogen without decomposing the compound. These N-unprotected derivatives appear to be ultra-sensitive to pH.





We also attempted (unsuccessfully however) to obtain S-heterosubstituted variants of N-thiolated beta-lactams as shown below, where the X group in the structure is either an O, S, or N. The way were tried to prepared these compounds is by N-thiolation using a sulfenyl halide as shown below. The sulfenyl halide was to be obtained by chlorination of the S,S'-heterosubstituted disulfide using either sulfuryl chloride or chlorine gas in tetrachloromethane. In neither instance were we able to prepare the desired sulfenyl chloride required for the attachment onto the beta-lactam. After considerable experimentation we gave up on this idea.



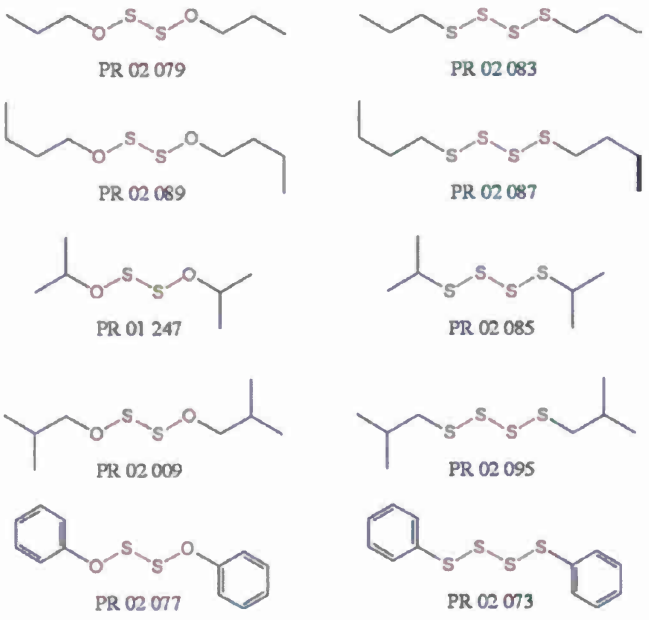


With a wide selection of N-thiolated beta-lactams prepared, we sent the compounds to the Anderson lab to conduct antibacterial screening against *Bartonella hensalae*. Disappointingly, the results indicated that none of the lactams possessed sufficient in vitro bioactivity to warrant further investigation. However, these compounds may be promising for certain bacteria, such as MRSA or

Bacillus, which have low intracellular glutathione levels, and this will be pursued independently. Due to these findings, a decision was made to abandon further studies of the beta-lactams for potential utility against *Bartonella* bacteria.

Sub-aim 2: Studies of S,S-heterosubstituted disulfides as small molecule antibiotic agents for use against Bartonella bacteria

In this second aim, we set out to examine S,S'-diheterosubstituted disulfides as potential small molecule growth inhibitors of *Bartonella* bacteria. These compounds were previously used in our attempted synthesis of S-heterosubstituted sulfenyl halides for derivatizing the beta-lactams in sub-aim 1 (above). These are intriguing molecules structurally given the contiguous arrangement of four heteroatomic centers. Our expectation from the outset of the study was that, like many natural products from garlic and anions and other plant sources, disulfide-containing small molecules often possess powerful antimicrobial properties that we thought could be effective against *Bartonella* bacteria. The advantage we thought these synthetic substances would have over natural product-derived compounds was that these could be chemically more stable (natural trisulfides for instance often oxidize rapidly in air), and the side chain groups (XR) could be easily varied to tune biological activity.

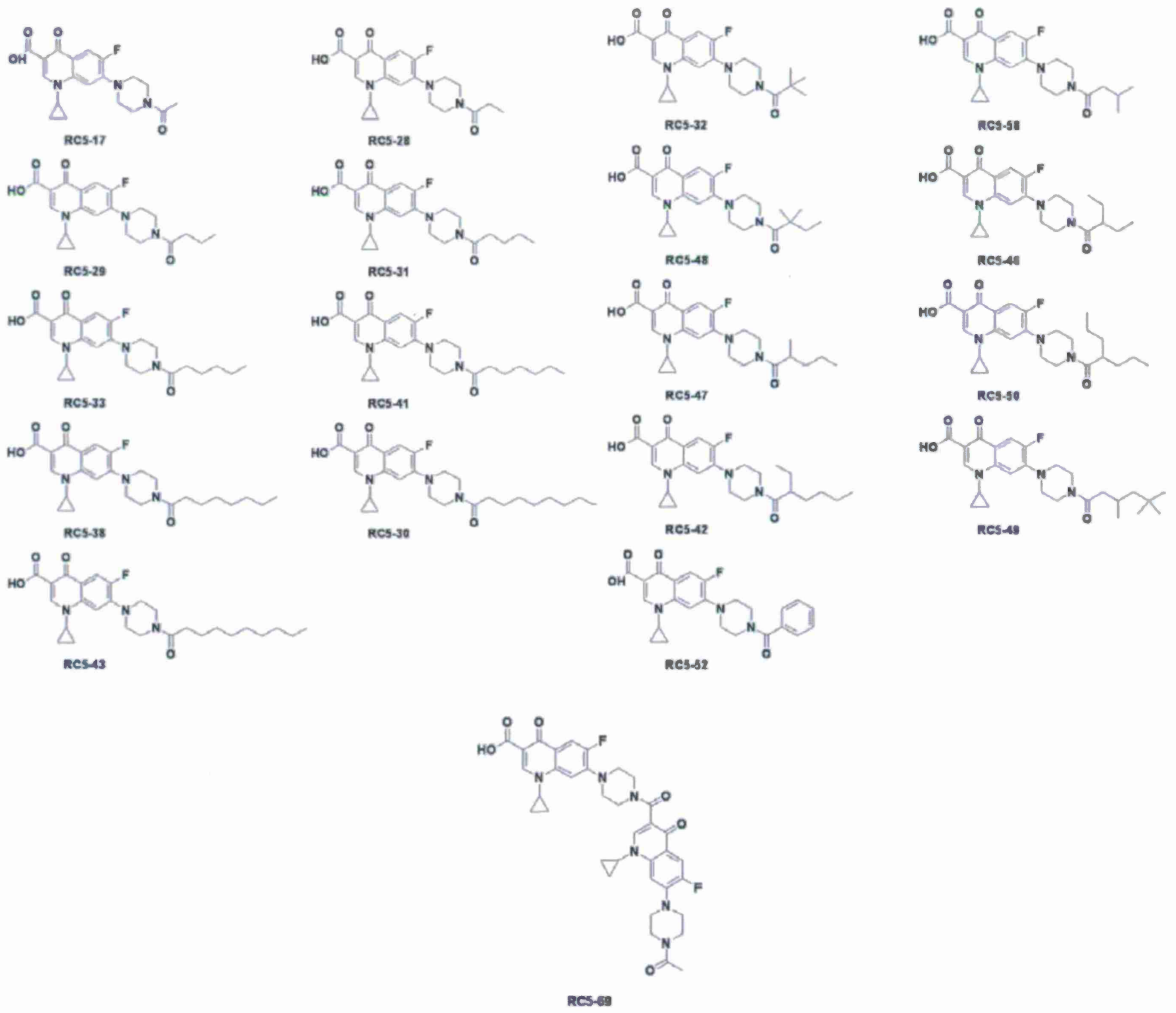


Sub-aim 3: Synthesis and biological evaluation of new N-acyl ciprofloxacins against Bartonella

In this aim, we explored the potential of N-acylated cipropfloxacins, a third family of small molecule antibiotics being developed in our laboratory, as anti-*Bartonella* compounds. These compounds were of interest for two reasons, first as antibacterial agents and then for potential incorporation into nanoparticles. The role of the N-acyl group was to increase cell membrane permeability compared to ciprofloxacin itself. The question we hoped to address was whether these

compounds could still exert powerful antibacterial properties, as a function of the N-acyl side chain (length, branching, and lipophilicity).

Ciprofloxacin Derivatives Synthesized for Microbiological Testing

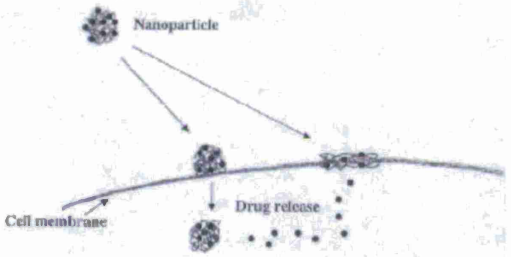


Ryan Cormier in my laboratory synthesized all of these cipro derivatives and confirmed their structure and purity by proton NMR spectroscopy. He then sent them to Burt Anderson's lab for in vitro testing.

Part II: Nanoparticles for targeting *Bartonella* bacteria

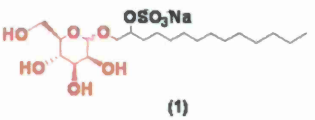
The second main subject of this study was the design and testing of new organo-based nanoparticles for targeting *Bartonella* bacteria. For these, we have examined a variety of different systems as nanoparticles, distributed in aqueous media, and their anti-*Bartonella* properties. The first of these is the poly(ethyl acrylate-styrene) nanoparticles previously reported from our lab which can be made by emulsion polymerization. We have likewise prepared antibiotic-conjugated variants in which a

penicillin or other beta-lactam antibiotic is covalently attached to the polymeric chain within the interior of the nanoparticle. The average size is about 45 nm, which is confirmed by dynamic lights scattering analysis. The working model we suggest for a possible mode of how these nanoparticles may attack bacteria is shown below. The nanoparticle, acting as an antibiotic carrier, interacts at the cell membrane and releases the antibiotic, either intracellularly after entering the cell (through some unknown mechanism) or directly at the cell membrane.

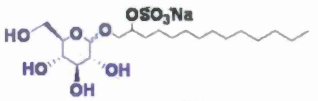


The image above on the right shows a transmission electron microscope image we captured of poly(ethyl acrylate-styrene) nanoparticles on the cell surface and inside a bacterial cell.

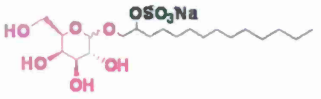
To target the nanoparticles to *Bartonella* bacteria, we attempted to synthesize new carbohydrate surfactants for nanoparticle formation, and to then study the properties of these nanoparticles in selectively targeting *B. henselae* cells in growth media. This was our first prototype of a "carbohydrate-coated" nanoparticle system for bacterial cell recognition and targeting. It entailed the synthesis of a glycosylated anionic surfactants synthesized from mannose (1), glucose (2) and galactose (3), shown below.



(1)

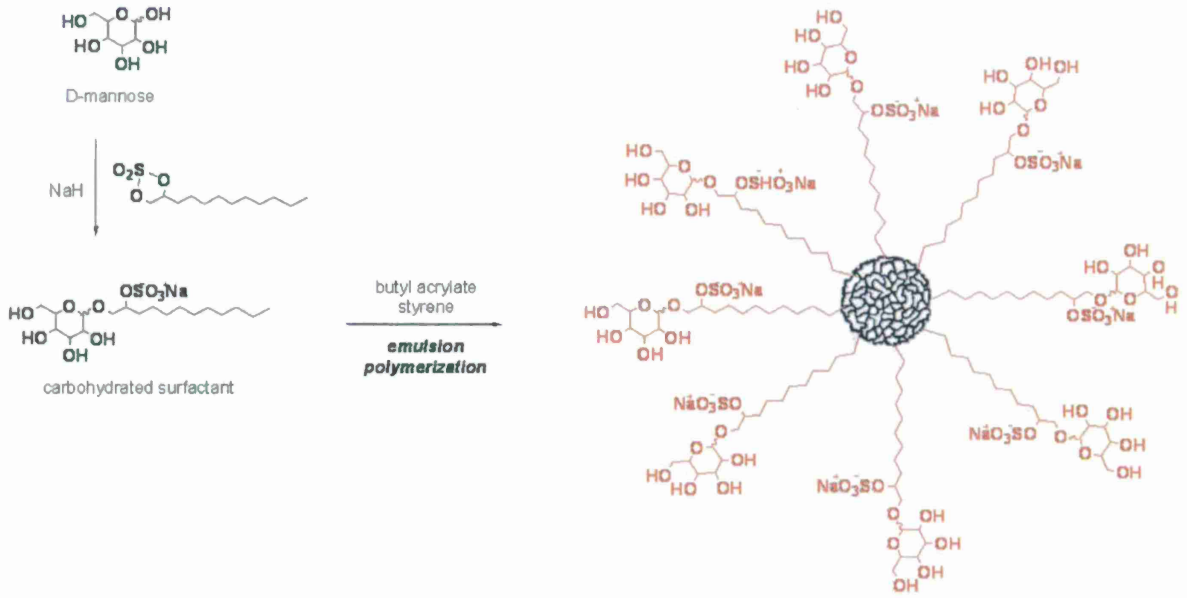


(2)



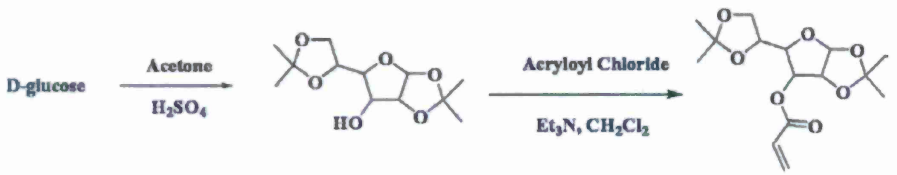
(3)

We attempted to carry out the emulsion polymerization of poly(ethyl acrylate-styrene) nanoparticles as detailed above, except replacing the SDS surfactant with one of the three glycosylated surfactant surrogates as shown below.



However, in no instance were we able to prepare satisfactory samples of the desired nanoparticle emulsions with these types of surfactants. The solutions that we obtained instead were not stable, and immediately led to precipitation of the material and with considerable amounts of gelling. Therefore, we explored alternatives, and one that worked well for us while using these glycosylated surfactants is shown below.

For reasons to be explained below, we were able to synthesize a new contruct for a polymer-based nanoparticles using a protected glucosyl acrylate shown below. This sugar acrylate monomer underwent emulsion polymerization using the earlier prescribed conditions, in which the original 7:3 ratio of ethyl acrylate and styrene monomers were replaced with the glucose-derived acrylate.



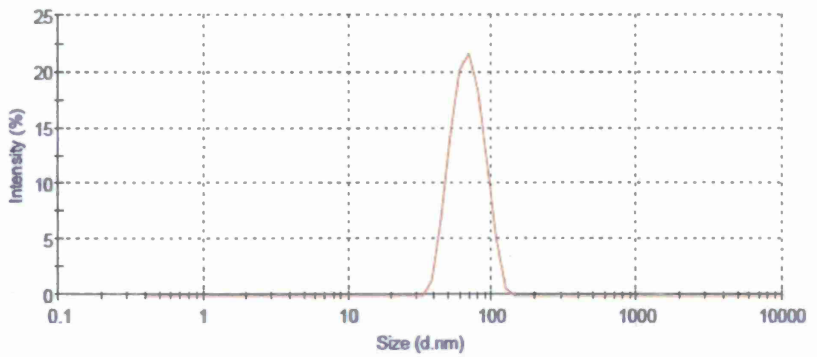
We were pleased to find that poly(glucosyl acrylate) nanoparticle emulsions were prepared at 70°C over three hours as shown below. The formulation used for the emulsion polymerization is listed below, and involves the use of 6 weight % of SDS as surfactant, and 2 weight % of potassium persulfate as radical initiator. For the procedure to work, we had to use methylene chloride to predissolve the acrylate to create the initial suspension for polymerization.

Glyconanoparticles were prepared with 3-O-acryloyl-1,2:5,6-di-O-isopropylidine-α-D-glucofuranose dissolved in CH_2Cl_2 by emulsion polymerization. Briefly, ultrapure water and sodium dodecyl sulfate (SDS) were added to the CH_2Cl_2 solution containing the monomer. The mixture was mixed with stirring under a nitrogen flow. A milky colored emulsion was formed by adding potassium persulfate to the mixture stirring at 70°C for 3 h.

Components	Amounts	Solid content (%)
3-*O*-acryloyl-1,2:5,6-di-*O*-isopropylidine-α-D-glucofuranose (In 1 mL of DCM)	200 mg	90.90
SDS	15 mg	6.81
Potassium persulfate	5 mg	2.27
Water	2 mL	

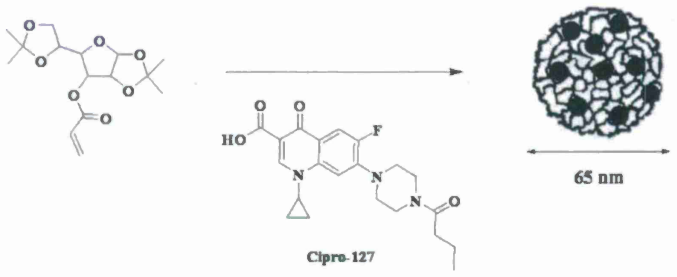
The particle sizes of the nanoparticles were measured by dynamic light scattering and to be very uniform, with a hydrodynamic diameter of about 65 nm (below).

Particle Size Distribution of Glucose-derived Nanoparticles



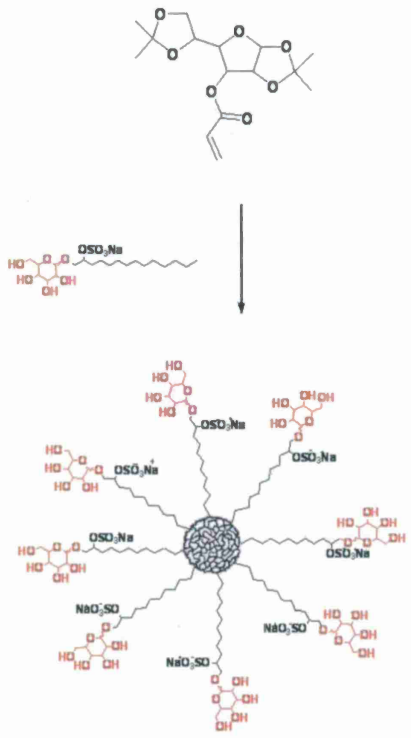
Similarly, we used this same procedure to prepare ciprofloxacin-containing nanoparticles. We note that our earlier attempts to incorporate ciprofloxacin itself into the matrix of poly(ethyl acrylate-styrene) nanoparticles were completely unsuccessful, but with the glucose-derived nanoparticles, we were able to obtain drug-entrapment when employing N-butyryl cipro derivative 127. We then sent these drug-free and drug-containing nanoparticles samples to Burt Anderson to assay for in vitro bioactivity against *B. henselae*.

Poly(glucosyl acrylate) Nanoparticles containing Cipro-127 (in water)



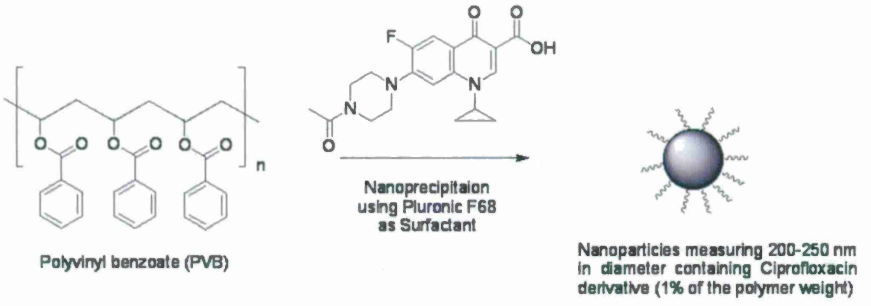
Components	Amounts	Solid content (%)
3-*O*-acryloyl-1,2:5,6-di-*O*-isopropylidine-α-D-glucofuranose (In 1 mL of DCM)	90 mg	90
Cipro-127	5 mg	5
SDS	3 mg	3
Potassium persulfate	2 mg	2
Water	4 mL	

Additionally, with the glucosyl acrylate, we found that the SDS could be replaced for one of the earlier-described saccharide surfactants, to give carbohydrate-surfactated nanoparticles based on the all sugar construct.

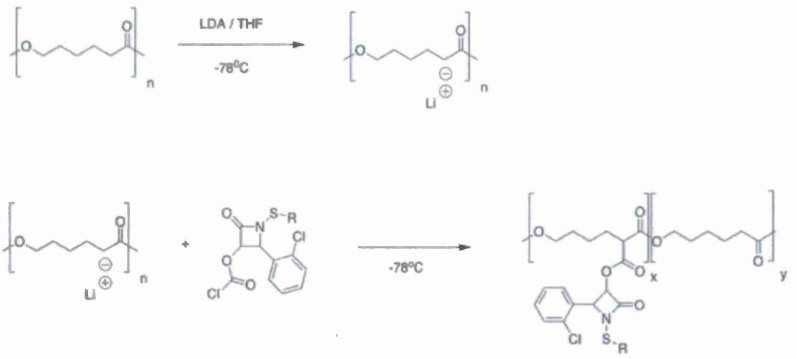


Signal interference due to FRET

As an alternative to polyacrylate nanoparticles prepared by emulsion polymerization, we synthesized poly(vinyl benzoate) nanoparticles by nanoprecipitation with and without a lipophilic probe (coumarin) to study stability and drug release. The advantage of this methodology was that the nanoparticle could be constructed from a polymer of known size through a process other than emulsion polymerization. The results of this study were published recently and a full description of the results can be found there ("Studies on the Preparation and in vitro Properties of Poly(vinyl benzoate) Nanoparticles for Molecular Delivery," Raphaël Labruère, Renaud Sicard, Ryan Cormier, Leigh West, and Edward Turos, *Journal of Controlled Release* **148**, 234-240 (2010)). The preparation and properties of poly(vinyl benzoate) nanoparticle suspensions as molecular carriers were thus described for the first time. These nanoparticles can be formed by nanoprecipitation of commercial poly(vinyl benzoate) in water using Pluronic F68 as surfactant, to create spherical nanostructures measuring 200-250 nm in diameter. These nanoparticles are stable in phosphate buffer and blood serum, and only slowly degrade in the presence of esterases. Pluronics stabilizes the nanoparticle and also protects it from enzymatic degradation. Addition of a lipophilic molecule such as coumarin 6 to the media allows for up to 1.6% of the polymer weight to be entrapped during nanoprecipitation, compared to a water-soluble compound (5(6)-carboxyfluorescein) which gave almost no loading. Kinetics experiments in phosphate buffer indicate that 78% of the coumarin 6 was encapsulated within the polymer matrix of the nanoparticle, and the residual surface-bond coumarin 6 was quickly released. The nanoparticles are non-toxic in vitro towards human epithelial cells and exert no observable bactericidal activity. These properties suggest that the poly(vinyl benzoate) nanoparticles may be suitable carriers for molecular delivery of lipophilic small molecules such as drugs and imaging agents



We also attempted to prepare samples of polyvinyl benzoate onto which an antibiotic is grafted directly onto the polymer strand. The attempts included deprotonating the commercial polyvinyl benzoate polymer under rigorously anhydrous conditions with lithium diisoprylamide in THF, followed by trapping of the anion with an acyl chloride prepared from the desired beta-lactam. However, these trials led to only minute amounts of the desired beta-lactam coupled product and not enough for us to work with to try to produce new nanoparticles containing the covalently-attached antibiotic. This appears to be a limitation of the nanoprecipitation method, in that while the polymer is premade to whatever length and specification that are required prior to nanoparticle assembly, the appending of a covalently-attached antibiotic onto the chain is not trivial.



Summary of Results

During this research period we have investigated the preparation and antimicrobial properties of two new families of antibacterial compounds, S,S'-hetero-substituted disulfides and N-acyl ciproploxacins. While the disulfides unfortunately do not possess very strong in vitro bioactive against the *Bartonella* species we examined. We hypothesize that this may be due to the nature of the cytosolic redox buffer within *Bartonella* bacteria. Our conclusion is that the compounds may not be worth pursuing further for anti-Bartonella applications. On the other hand, new N-acyl ciprofloxacin analogues we have prepared are powerful growth inhibitors of *Bartonella* and thus represent a new family of antibacterial agents for *Bartonella*, We observe that in vitro bioactivity decreases with increasing lipophilicity of the N-acyl side chain. Finally, surface-glycosylated polyacrylate nanoparticles derived from a protected glycosylated acrylate monomer were developed and found to have cell targeting capabilities for *Bartonella* that may be useful for detection, antigrowth applications, or molecular delivery.

Reasons Why Goals Were Not Met

Inevitably, in projects involving organic synthesis, unanticipated issues often appear during the course of the investigations that prevent one from achieving the full potential of the project. That was certainly the case here. Below is a brief description of some of the things we encountered, first in terms of obstacles that we were able to overcome, and then those that we could not overcome as the project period closed.

Obstacles Encountered and Overcome

- Initially, we observed quite unexpectedly that some (but not all) of the S,S'-heterosubstituted disulfides were found to be *unstable* in DMSO solution. Through experimentation, we ultimately overcame this by preparing fresh samples of the disulfided immediately prior to the microbiological testing.
- Another problem we faced early on in the studies was the synthesis and stability of glycosylated nanoparticles- the poly(butyl acrylate-styrene) nanoparticles were difficult to make with surface-bound carbohydrates on the surface. We overcame this by developing an entirely new poly(glucose)-based nanoparticle construct which has on its outer surface one or more *glycosylated surfactant* moieties.

Obstacles Encountered Which Yet Remain Unanswered

- The synthesis of N-(S0heterosubstituted) beta-lactams could not be achieved due to technical difficulties in the synthesis.
- The N-thiolated beta-lactams and S,S'-heterosubstitutd disulfides were found not to be especially active against *Bartonella*, and we abandoned these studies as a result.
- Recently, for the intention of publication, we repeated some of the agglutination studies using new batches of glycosylated nanoparticles, and found unexpectedly that these do not retain their powerful agglutination properties. We think this is due to changes in the microbes, not to the nanoparticles, but as of yet have not been able to definitively figure out the basis for this problem.
- Finally, we were unable to attach antibiotics directly to the polymerized framework of commercial poly(vinyl benzoate) in sufficient amounts to prepare nanoparticles derived from this building block.

Students and Postdoctorals who Worked on These Projects in the Turos Lab

Postdocs: Dr. Renaud Sicard, Dr. Sampath Abeylath, Dr. Raphael Labreure
Graduate students: Praveen Ramaraju, Ryan Cormier, Biplab Bhattacharya
Lab technician: Danielle Gergeres

Intellectual Property Developed

Provisional Patent Application filed on November 18, 2010; "Poly(vinyl benzoate) Nanoparticles for Molecular Delivery," Edward Turos, Raphael Labreure, Renaud Sicard and Ryan Cormier, Disclosure filed November 1, 2010

U.S. Utility National Patent Application filed February 22, 2011: "Activity of New N-Acylated Ciprofloxacin Derivatives Against Facultative Intracellular Bacteria," Edward Turos, Ryan Cormier, Burt Anderson, John Thomas, Rebecca Kapolka, and Glenn Roma; Provisional Patent Application filed on February 19, 2010; "Bartonella as a Model Intracellular Pathogen for Developing Therapeutics," Disclosure filed on February 18, 2010 .

Poster Presentations

"Development of Bacterial Adhesin-Specific Polymer Glyconanoparticles as a Non-Antibiotic Treatment for Bacterial Infections," Sampath C. Abeylath, Danielle Gergeres, Edward Turos, *2008 Symposium on Detection, Diagnostics, and Therapeutics*, Florida Center of Excellence in Biomolecular Identification and Targeted Therapeutics Research Symposium, October 15-17, 2008

"N-Thiolated β-Lactams: Altering Microbiological Activity and Bacterial Cell Targeting with C3 Ring Functionality," Biplob Bhattacharya, Katie Prosen, and Edward Turos, *Graduate Student Research Symposium*, Department of Chemistry, University of South Florida, FL, October 8, 2009

"Synthesis and Antibacterial Activities of *S*-Heterosubstituted Disulfides," Praveen Ramaraju, Danielle Gergeres, and Edward Turos, *2009 Symposium on Detection, Diagnostics, and Therapeutics*, Florida

Center of Excellence in Biomolecular Identification and Targeted Therapeutics Research Symposium, University of South Florida, Tampa, FL, October 15, 2009

"N-Thiolated β-Lactams: Altering Microbiological Activity and Bacterial Cell Targeting with C3 Ring Functionality," Biplob Bhattacharya and Edward Turos, *2009 Symposium on Detection, Diagnostics, and Therapeutics*, Florida Center of Excellence in Biomolecular Identification and Targeted Therapeutics Research Symposium, University of South Florida, Tampa, FL, October 15, 2009

"*Bartonella*; a Model Intracellular Pathogen for Developing Therapeutics" John C. Thomas, Rebecca J. Kapolka, Ryan Cormier, Edward Turos and Burt E. Anderson, *23rd Meeting of the American Society for Rickettsiology*, Hilton Head Island, SC, August 2009

"Activity of New N-Acylated Ciprofloxacin Derivatives against Facultative Intracellular Bacteria," John C. Thomas, Rebecca J. Kapolka, Ryan Cormier, Glenn Roma, Edward Turos, and Burt E. Anderson, *American Society of Microbiologists Biodefense Meeting*, Baltimore, MD, February 21-25, 2010

"N-Thiolated β-Lactams: Altering Microbiological Activity and Bacterial Cell Targeting with C3 Ring Functionality," Biplob Bhattacharya and Edward Turos, *American Chemical Society National Meeting*, San Francisco, CA, March 21-25, 2010

N-Acylated Ciprofloxacins," Kornwalee Wiangkham, Ryan Cormier, and Edward Turos, *Eighth Annual Raymond N. Castle Student Research Conference*, University of South Florida, April 15, 2010

"N-Thiolated β-Lactams: Altering Microbiological Activity and Bacterial Cell Targeting with C3 Ring Functionality," Biplob Bhattacharya, Silvia Robles, and Edward Turos, *Eighth Annual Raymond N. Castle Student Research Conference*, University of South Florida, April 15, 2010

"N-Thiolated β-Lactams: Altering Microbiological Activity and Bacterial Cell Targeting with C3 Ring Functionality," Biplob Bhattacharya and Edward Turos, *Florida Annual Meeting and Exposition*, Innisbrook Resort and Golf Club, Innisbrook, FL, May 13-15, 2010

Manuscript Submitted

Burda, W., R. Cormier, L. Harrington, J. Edlinger, K.M. Kodigepalli, J. Thomas, R. Kapolka, G. Roma, B.E. Anderson, E. Turos, and L.N. Shaw. Antimicrobial properties of N-acylated ciprofloxacin derivatives. Submitted.

Final Report

Anderson Subproject

The goals of this project center around the emerging pathogen *Bartonella henselae*. The first aim includes efforts to harness the type IV secretion system for the delivery of proteins to host endothelial cells. The long-term goal would be utilize this system to deliver proteins for gene therapy or even vaccination. The second aim is to test gene-specific peptide nucleic acids for their ability to inhibit gene expression and ultimately growth of *Bartonella* spp. The final aim revolves around the testing of novel antibiotics and antibiotic-nanoparticles complexes for anti-bacterial activity against *B. henselae*.

Aim 1: Evaluate the VirB/Type IV secretion system of *B. henselae* to deliver proteins into target cells

- *construct BEP-reporter gene fusions, insert behind Trc promoter of pNS2Trc*
- *electroporate into B. henselae*
- *monitor reporter gene expression/translocation into HMEC-1*

Toward the goals of this aim, the genes for two Bartonella effector proteins (Beps) were cloned into the *Bartonella* shuttle vector pNS2Trc. The *B. henselae* genes encoding Bep A and BepB were directionally cloned behind the strong Ptrc promoter. The resulting recombinant fusion proteins consisted of a N-terminal green fluorescent protein domain (Gfpmut3) and a C-terminal Bep (either BepA or BepB)(**Fig. 1**). Since Beps are translocated by the *B. henselae* type IV secretion system it was reasoned that all or part of the Beps could be used to help deliver proteins to the target host cell cytoplasm. The gfpmut3 reporter gene was used to test this hypothesis. After obtaining the recombinant plasmids they were transformed into *B. henselae* via electroporation using kanamycin for selection. The resulting strains of

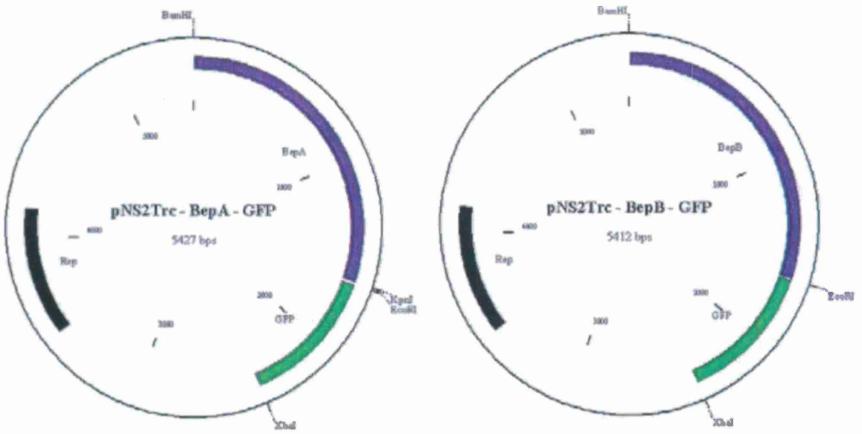


Figure 1. Recombinant plasmids encoding for BepA- or BepB-green fluorescent protein fusions. The backbone consists of *Bartonella* shuttle vector pNS2Trc. The plasmids were transformed into the Houston-1 strain of *B. henselae*.

B. henselae were cultured with human endothelial cell line HMEC-1 to determine if the fusion protein was translocated into the cytoplasm of HMEC-1. A positive result would provide evidence that the Type IV secretion system of *B. henselae* could be used to deliver proteins consisting of the desired protein fused to one of the Beps. Confocal microscopy was used to assess the amount of the gfpmut3 genes that was expressed in the HMEC-1 cells. Most cells can be seen to express gfpmut3 in amounts that appear as faint green fluorescence (**Fig. 2A**). Unfortunately, it could not be determined if this fluorescence is due to translocated BepA or if the entire bacterial cell is invading the HMEC-1 cells. This issue would need to be resolved in order to proceed with attempts to fuse the gene of any protein for delivery into endothelial cells. In addition, it may be desirable to restrict the bep coding region to only that which codes for the intracellular delivery domain.

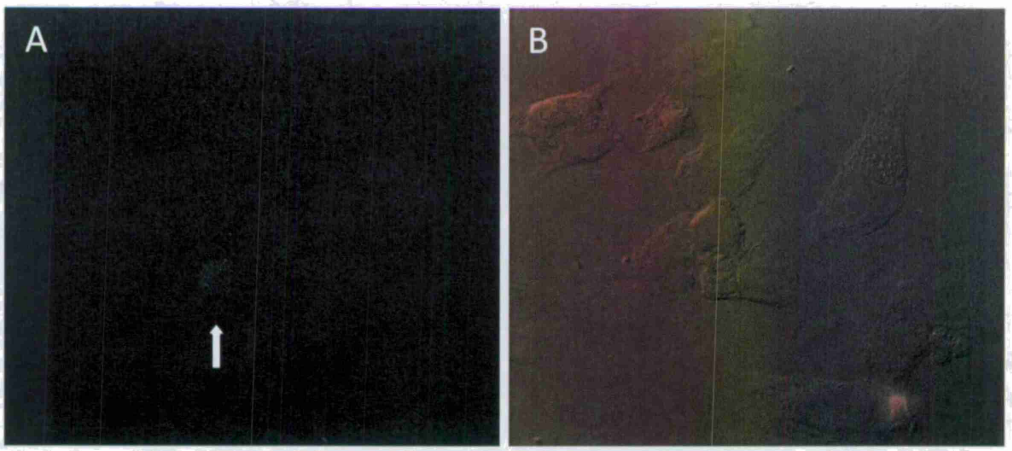


Figure 2. Fluorescence microscopy showing (A) gfp expression in HMEC-1 cells infected with *B. henselae* with pNS2Trc-BepA-Gfp. (B) visible light microscopy showing same infected HMEC-1 cells. Arrow indicates an area with green fluorescence.

Aim 2: Test PNAs designed to silence bacterial gene expression in *B. henselae*

- *synthesize PNAs against candidate genes (batR, ompR, badA, virB2)* **(Mclaughlin)**
- *electroporate into B. henselae*
- *examine gene/protein expression*
- *alter PNA R-groups to alter membrane permeability*
- *examine PNA-R for intracellular silencing of B. henselae genes*

Unfortunately, we were unable to synthesize gene-specific peptide nucleic acids (see Mclaughlin report). Accordingly, no PNAs were tested and the goals of this aim were not achieved.

Aim 3: Test antibiotics for activity against the model intracellular bacterium *B. henselae*

- *synthesize antibiotics (***Turos***)*
- *semi-quantitative testing of antibiotics via Kirby-Bauer disk diffusion*
- *perform MIC testing using agar dilution method*
- *assess the activity of antibiotics against intracellular bacteria*

In collaboration with the Turos lab, three types of antibiotics/nanobiotics were tested for activity against *Bartonella* species. These included carbohydrate-surfacted nanoparticles,N-thiolated beta-lactams , and N-acyl ciprofloxacin derivatives (see Turos report for details). The N-acyl ciprofloxacin compounds were found to have the greatest activity against *Bartonella* species and were shown to have considerable acitivity against intracellular *B. henselae*.

Glyconanoparticles

To investigate the potential interaction of the carbohydrate-surfactated nanoparticles with *Bartonella* bacteria, transgenic *Bartonella henselae* Houston-1 rough strain containing red fluorescent protein was used. This enabled the cells to be easily visualized under a fluorescent microscope.

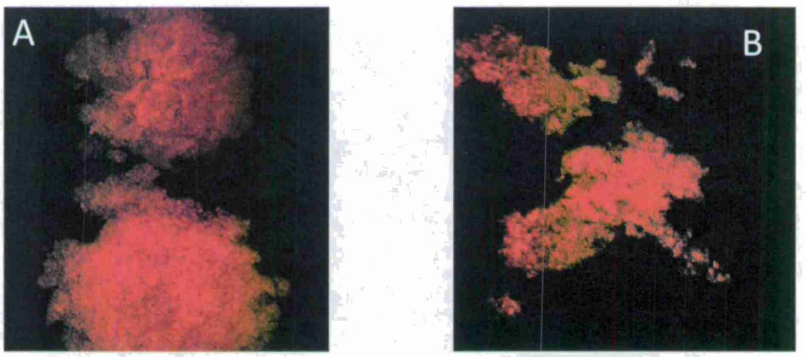


Figure 3. Glyconanoparticle induces agglutination of *B. henselae* expressing red fluorescent protein (BSred2). **(A)** Mannose containing glyconanoparticle sample 102 at 0.3 µg/ml **(B)** bacteria alone showing autoagglutination.

With the mannose-surfactated nanoparticle emulsion, the nanoparticles induced agglutination of *B. henselae* within 15 minutes at a concentration of 0.3 ug/ml of the nanoparticle in the bacterial growth media (**Fig. 3**). The images above show that the nanoparticle-induced agglutination is distinct to that resulting from self-agglutination of the cells in the absence of nanoparticles, which *Bartonella* undergoes relatively readily. The process appears to be more rapid and morphologically controlled in the presence of the nanoparticle, which we believe suggests that the mannose-binding receptors on the surface of the bacterial cells may be interacting strongly with the mannose surfactants bound to the surface of the nanoparticles. Interesting, we found that the agglutinated cells take on a green coloration in the presence of the fluorescein-labeled nanoparticles, due to co-localization and what may be signal interference arising from a FRET effect (**Fig. 4**). This suggests close interaction between the nanoparticles and the agglutinated cells, and a strong likelihood that the mannose-containing nanoparticles do bind to the target cells. As the grant period was ending, we wanted to follow this up with more studies to determine if there was a linear dose response for cell agglutination, or if other types of monosaccharide surfactants on the surface of the nanoparticles would cause cell agglutination as well. These studies were not conducted, however, due to lack of time. A fluorescently-labeled (fluorescein) variant of the mannose-surfactated nanoparticles prepared above were incubated with RFP *Bartonella* and then viewed by fluorescence microscopy.

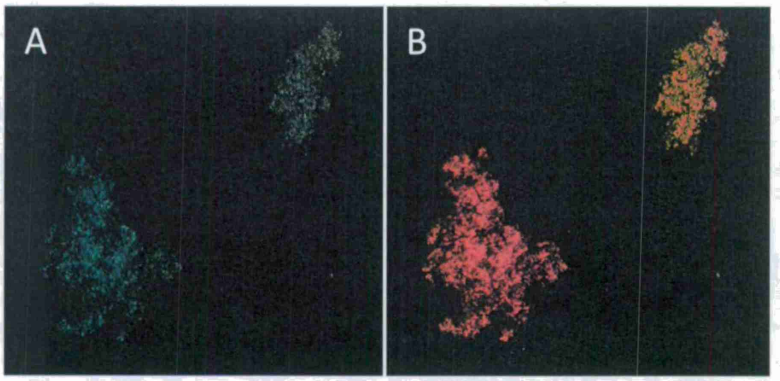


Figure 4 (A). Co-localization of fluorescein-labeled mannose containing glyconanoparticle (sample 102) and **(B)** red fluorescent protein expressing *B. henselae*.

To determine if pre-incubation of glyconanoparticle 102 with *B. henselae* could prevent attachment and internalization of the bacteria into human endothelial cells an invasion assay was performed. Bacteria and glyconanoparticles were incubated for 60 minutes and the bacteria were then incubated with the HMEC-1 human microvascular cell line. After infection for an additional hour, the cells were washed 2x with PBS then treated with gentamicin (200 µg/ml) for 1 hour to kill extracellular adherent bacteria. After incubation of the infected cells for 48 hours, the cells were lysed with 0.1 % saponin and the number of viable intracellular bacteria were determined by plating on culture medium (**Fig. 5**). There appears to be no inhibition of cell attachment or invasion by preincubation of the glyconanoparticle with bacteria suggesting that bacterial attachment to host endothelial cells is not mannose-dependent.

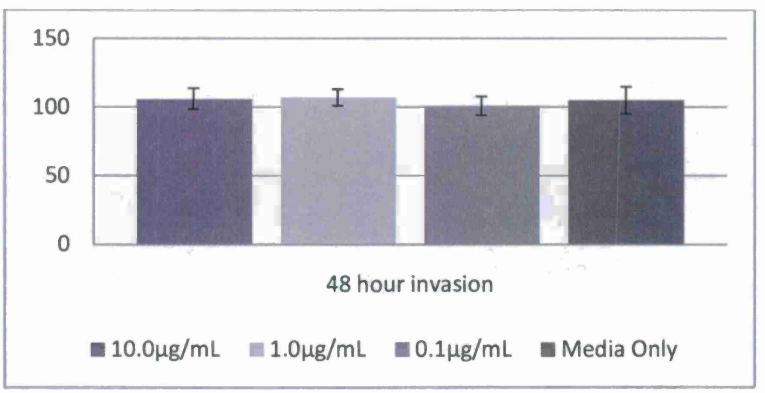


Figure 5. Cell invasion assay of HMEC-1 cells and bacteria preincubated with varying concentrations of glyconanoparticle sample 102. The number of viable bacteria from HMEC-1 cell lysates is shown.

N-thiolated beta-lactams

Compounds synthesized by the Turos lab were examined for the *in vitro* antibacterial properties of these disulfides against *Bartonella* bacteria. This was done against nine species and strains of *Bartonella* using a modified Kirby-Bauer disk diffusion assay on agar plates. *Bartonella* strains were

cultured on chocolate agar prepared from heart infusion agar base supplemented with 5% bovine hemoglobin at 37 °C in 5% CO_2. Disk diffusion assays for antimicrobial activity were performed in triplicate. Twenty µl of relevant antibiotics, at a concentration of 1mg/ml, were spotted onto the center of 6 mm paper disks (BBL) on a sheet of aluminum foil, in a biological safety cabinet. Disks were allowed to dry for 20 minutes, and then stored in a sealed bag with desiccant at 4 °C. Growth from 4 day old plates was resuspended in 1.0 ml sterile HIB, and turbidity was adjusted to a McFarland 2.0 by visual inspection. The bacterial suspension was spread over the surface of a chocolate agar plate using a swab. The inoculum was allowed to dry into the agar in a biological safety cabinet for 15 minutes. Using fine point forceps, antibiotic containing disks were carefully placed in the center of the plate, which were then inverted and incubated at 37 °C in a 5% CO_2 incubator for one week. For all organisms, the zone of inhibition was measured by recording the diameter to the nearest mm for each disk (table below). The data show that the compounds have relatively weak antimicrobial activity against the various *Bartonella* species tested.

Anti-Bartonella Activities

Zone diameter (in mm)

Compound	B. henselae (Houston-1)	B. henselae (SD-2)	B. elizabethae	B. quintana (Fuller)	B. quintana (D-Perm)
A (PR_02_77)	6	6	6	6	6
A' (43)	35	48	50	41	45
C (PR_02_83)	6	10	12	11*	11
D (PR_01_27)	8	14	24	6	21
E (PR_02_89)	6	6	6	10	6
F (133)	9*	8	10*	6	6
A' (123)	65	62	56	8	47
B' (PR_02_73)	14	14	15	6	22
C' (PR_02_95)	6	6	6	12*	13
D'(PR_02_009	6	6	6	6	6
E' (PR_02_85)	6	6	6	6	11*
F' (PR_02_79)	6	6	6	6	6
Rifampin	50	50	52	37	40
DMSO	6	6	6	6	6

*Weak inhibition exhibited

Nanobiotic	B. henselae (Houston-1 "Rough")	B. quintana U-Mass	B. henselae Marseille	B. henselae SA-1
A (PR_02_77)	6	6	6	6
B (145)	34	24	38	30
C (PR_02_83)	6	12	10	6
D (PR_01_247)	12	25	12	11
E (PR_02_89)	6	6	6	6
F (133)	8	6	7*	7*
A' (125)	52	41	61	52
B' (PR_02_73)	17	6	17	8
C' (PR_02_95)	6	6	8	6
D' (PR_02_009)	6	6	6	6
E' (PR_02_85)	6	6	10	6
F' (PR_02_79)	6	6	6	6
Rifampin	62	53	22	68
DMSO	6	6	6	6

N-acyl ciprofloxacin derivatives

Zones of inhibition for N-acyl ciprofloxacin derivatives versus 10 different *Bartonella* strains were measured by disk diffusion as with the N-thiolated beta-lactam compounds. Obviously, the N-acyl ciprofloxacin derivatives demonstrated much greater antibacterial activity against *Bartonella* species than did the N-thiolated beta-lactam compounds.

Antibiotic Activity

Zones of inhibition measured in mm

	B. henselae (Houston-1 "Smooth")	B. henselae (SD-2)	B. elizabethae	B. quintana (Fuller)	B. quintana (D-Perm)	B. henselae (Houston-1 "Rough")	B. henselae (SA-1)	B. henselae (Marseille)	B. quintana (UMass)	B. vinsonii
28	56	66	53	58	54	74	38	63	45	69
29	34	46	42	29	42	60	40	45	38	44
30	6	12	12	11	7	8	12	11	10	14
31	38	47	35	35	35	42	54	58	6	57
32	40	41	36	33	38	61	38	42	24	36
33	31	38	27	30	24	35	24	6	6	37
38	16	16	14	14	14	12	8	13	6	15
41	28	25	22	20	13	22	26	26	18	24
42	12	13	12	6	6	19	11	12	6	15
43	10	10	9	11	6	7	9	7	6	9
46	33	29	25	17	13	26	31	30	17	25
47	37	33	32	24	15	33	34	39	20	30
48	41	37	32	27	17	30	32	37	22	33
49	15	15	8	7	6	11	11	11	9	10
50	14	11	6	6	6	9	12	9	6	6
52	21	18	13	7	6	13	17	13	13	12
58	62	54	37	36	32	43	49	38	44	40
69	43	40	30	17	16	35	39	27	38	29
143	50	58	34	27	27	51	52	38	6	38
147	56	56	37	30	32	55	58	42	6	45

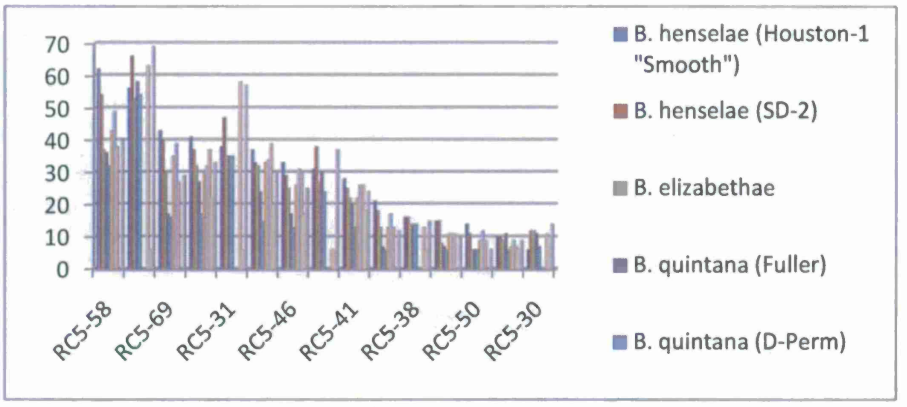


Figure 6. Zone of inhibition sizes (in mm) for Ciprofloxacin derivatives against different *Bartonella* species

The data in the chart above (**Fig. 6**) have been organized to show the general trend of lipophilicity and its effect on anti-*Bartonella* bioactivity. In the chart, lipophilicity increases from left to right on the x-axis, with zones of growth inhibition (bioactivity) given on the y-axis. The data shows anti-*Bartonella* activity is inversely proportional_to drug lipophilicity. This data is being reported in a manuscript that was submitted for publication.

In addition to the Kirby-Bauer disk diffusion studies, we tested these compounds for determining the minimum inhibitory concentrations (MICs) using the agar dilution method. *Bartonella henselae*, *Bartonella quintana*, *Bartonella elizabethae* and *Bartonella vinsonii* strains were tested for growth on chocolate agar plates containing antibiotics at 10.0 µg/ml, 1.0 µg/ml, and 0.1 µg/ml. Compounds inhibiting growth at≤ 1.0 µg/ml were further tested to determine the exact MIC by agar dilution using 2-fold dilutions at and below 1.0 µg/ml. Agar plates containing DMSO (without compound) as a control were prepared at the highest dilution to assess any anti-bacterial activity associated with the solvent. Growth from four day old chocolate agar plates was collected for each *Bartonella* strain tested. The growth was suspended into 0.5 ml of sterile Heart Infusion broth. The

MIC's determined by agar dilution against *B. henselae* Houston-1. Results are shown as growth (G) or no growth(NG) at each drug concentration.

	10.0µg/mL	5.0µg/mL	1.0µg/mL	0.8µg/mL	0.5µg/mL	0.2µg/mL	0.1µg/mL
28	NG	NG	NG	NG	NG	G	G
29	NG	NG	NG	NG	NG	G	G
30	NG	G	G	G	G	G	G
31	NG	NG	G	G	G	G	G
33	NG	NG	G	G	G	G	G
38	NG	G	G	G	G	G	G
41	NG	G	G	G	G	G	G
43	NG	NG	G	G	G	G	G
46	NG	NG	G	G	G	G	G
47	NG	NG	NG	NG	G	G	G
48	NG	NG	NG	NG	NG	G	G
49	NG	NG	NG	G	G	G	G
50	NG	NG	G	G	G	G	G
58	NG	NG	NG	NG	NG	NG	G
69	NG	NG	NG	NG	NG	NG	G

turbidity was adjusted to a McFarland 2.0 by visual comparison to turbidity standards. Twenty five µl of each bacterial suspension were spotted onto each plate containing varying concentrations of drug. Chocolate agar plates with no antibiotics were used as controls to confirm viability. Inoculation drops were allowed to briefly dry into the agar. Plates were inverted and incubated at 37 °C with 5% CO_2 for 7 days. Growth was recorded for each strain on each of the duplicate plates. The most active compounds were RC5-58 and RC5-69, with MIC values at or below 0.2 ug/ml (Table above).

To assess the ability of select representative N-acyl ciprofloxacin derivatives for activity against intracellular *B. henselae* an infection experiment was performed. The HMEC-1 human microvascular endothelial cell line was maintained in MCDB131 medium supplemented with 10% FBS, 5% L-glutamine, 10 ng/ml EGF, and 1 µg/ml hydrocortisone. HMEC-1 were infected with the Houston-1 strain of *B. henselae* for 4 hours. After infection, the cells were washed 2x with PBS then treated with gentamicin (200 µg/ml) for 1 hour to kill extracellular adherent bacteria. Infected cells were washed as before and media with test antibiotics were added at concentrations of 0.1 µg/ml, 1.0 µg/ml, 10 µg/ml. After addition of test antibiotics, infected cells were incubated for 96 hours. Following incubation, the antibiotics were removed, the infected cells were washed as before, and lysed with 0.1% saponin. Lysates were plated on chocolate agar and incubated for 7 days. The number of colony forming units (CFU's) were counted to determine the number of viable bacteria. The cell survival results for 96 hour incubation time using 1.0 µg/mL of test compound are shown below for five of the cipro analogues. Values are shown as percentage of CFUs in comparison to the control which contained only media.

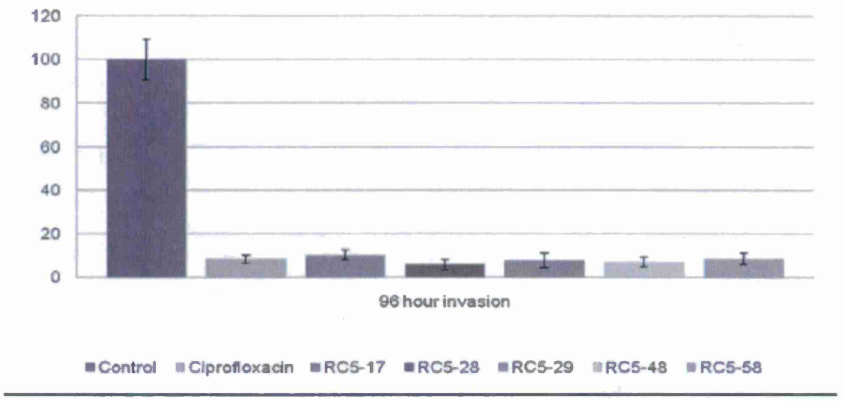


Figure 7. Activity of N- acyl ciprofloxacin derivatives against intracellular *B. henselae*. HMEC-1 cells were infected with the Houtson-1 strain of *B. henselae* and cultured in the presence of the compounds for 96 hours. The infected cells were lysed and the number on viable bacteria determine by plating compared to a control of DMSO (100%).

Additional experiments were performed with compounds RC5-28 and RC5-29 to further test their activity against intracellular bacteria. Experiments were performed as before but with three different concentrations (10.0 µg/ml, 1.0 µg/ml and 0.1 µg/ml) of the test compound. Again, the results show that these compounds, especially RC5-29 were able to gain access and kill intracellular *B. henselae* even at concentrations as low as 1.0 µg/ml (**Fig. 8**).

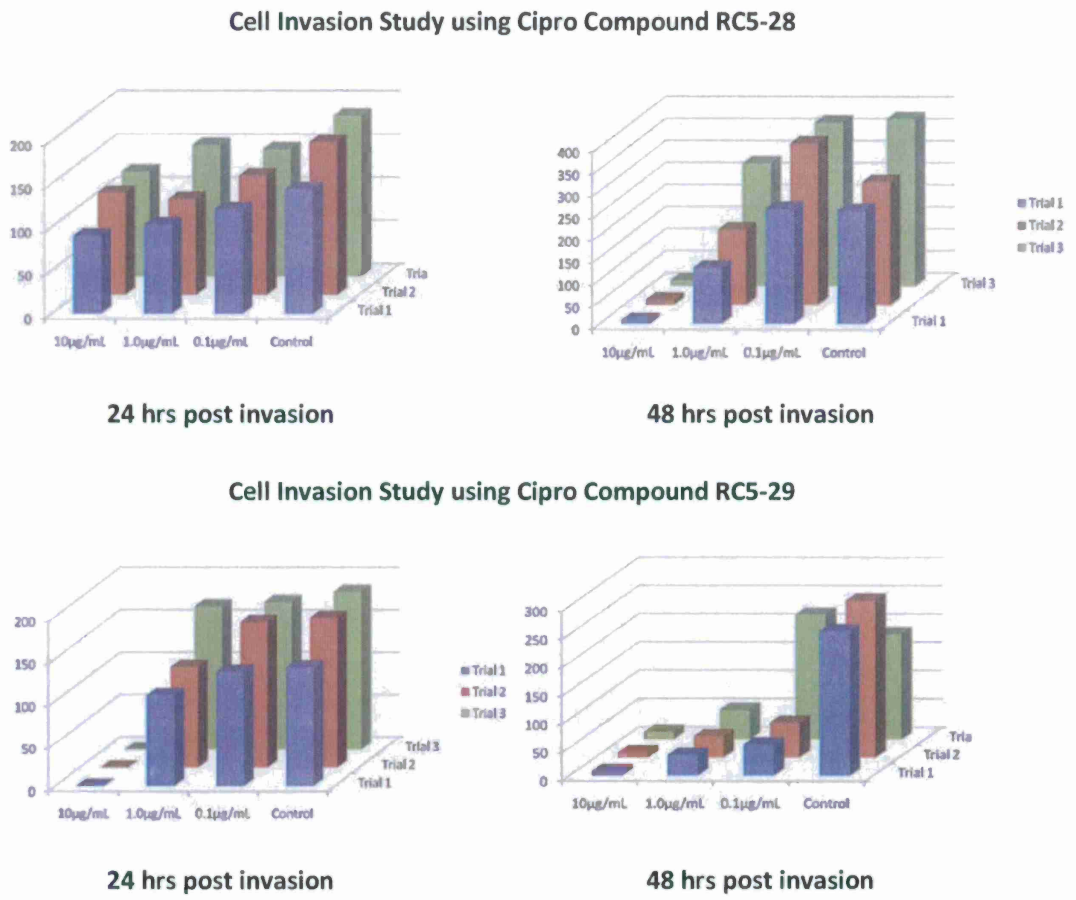


Figure 8. Activity of N- acyl ciprofloxacin derivatives at varying concentrations against intracellular *B. henselae*.

Given that fluoroquinolones target enzymes that mediate supercoiling, such as DNA gyrase and Topoisomerase IV, we employed a classic biochemical assay to measure gyrase activity in the presence of select compounds using purified gyrase from *E. coli*. Activity of select test drugs against DNA gyrase was tested using relaxed circular pUC19 DNA in the presence of *E. coli* DNA gyrase and antibiotics at concentrations of 1 μg/ml, 5 μg/ml, 10 μg/ml, and 25 μg/ml. Samples were incubated at 37 °C for 1 hour then analyzed by gel electrophoresis to quantify the amounts of relaxed and supercoiled DNA. Test compounds all exhibited clear activity in inhibiting the ability of DNA gyrase to initiate supercoiling of pUC19 at concentrations ranging from 1.0 to 100 μg/ml (**Fig. 9**). Thus, it appears that all five test compounds have inhibitory activity against DNA gyrase from *E. coli* and likely act through a mechanism identical to other fluoroquinolones. Our studies also indicate that N-acylated ciprofloxacins inhibit DNA gyrase in the same manner as ciprofloxacin, indicating that modification of the piperazinyl nitrogen by acylation does not alter how the molecule functions toward its bacterial target.

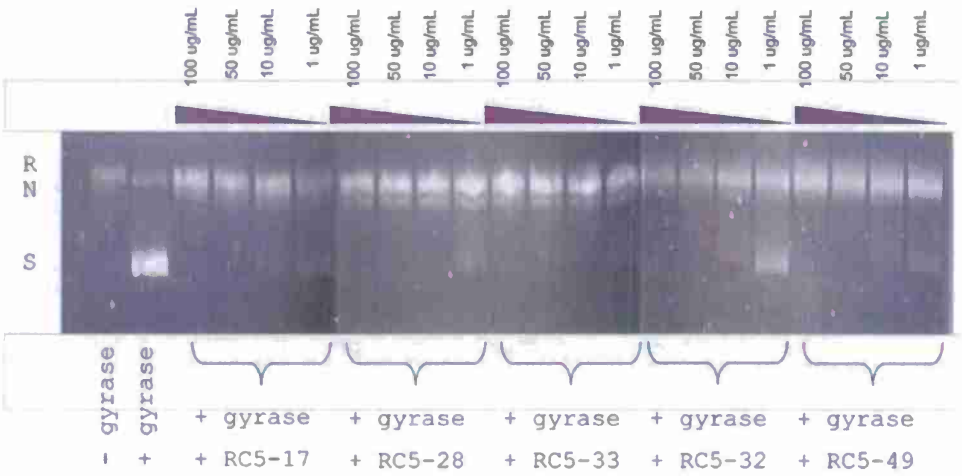


Figure 9. Inhibition of DNA gyrase by select N-acyl ciprofloxacin derivatives. Relaxed plasmid (R) DNA incubated for 1 hour in labeled conditions. Presence of gyrase alone caused supercoiling of DNA leading to a shift on the gel to a supercoiled form (S). Addition of ciprofloxacin derivatives at decreasing concentrations (100ug/mL, 50ug/mL, 10ug/mL, 1ug/mL) demonstrated inhibition of gyrase activity as shown by lack of shift. At lower concentrations, (10ug/mL, 1ug/mL), shift again begins to be seen.

Summary of Results

Our studies that attempted to utilize the type IV secretion system of *B. henselae* as a possible tool for gene/protein delivery yielded inconclusive results in which we were unable to confirm protein translocation as opposed to bacterial infection. We believe the potential for this system remains viable and worthy of future study. Our experiments with peptide-nucleic acids were not initiated since there was no successful synthesis of these molecules. During this research period we have investigated the antimicrobial properties of two new families of antibacterial compounds, S,S'-hetero-substituted disulfides and N-acyl ciproploxacins. The disulfides unfortunately do not possess very strong in vitro bioactive against the *Bartonella* species we examined and we conclude that the compounds may not be worth pursuing further for anti-Bartonella applications. On the other hand, new N-acyl ciprofloxacin analogues were shown to be powerful growth inhibitors of *Bartonella* and thus represent a new family of antibacterial agents for *Bartonella* and perhaps other intracellular bacteria as well. We observe that in vitro bioactivity decreases with increasing lipophilicity of the N-acyl side chain. Finally, surface-glycosylated polyacrylate nanoparticles derived from a protected glycosylated acrylate monomer were developed and found to have cell targeting capabilities for *Bartonella* that may be useful for detection, antigrowth applications, or molecular delivery.

Reasons Why Goals Were Not Met

The unanticipated issue concerning the delivery of BepA-Gfp and BepB-GFP was one that could not have been predicted in advance and prevented us from demonstrating using a Gfp reporter that the *B. henselae* type IV secretion system could be used for targeted delivery. It might be possible that using more sophisticated methods translocation could have been demonstrated. Likewise, the inability to

synthesize PNAs for *B. hensleae* genes was disappointing but was due to difficulties in their synthesis. In contrast our goals to test the antimicrobial activity of the compounds synthesized by Dr. Turos were largely achieved.

Obstacles Encountered and Overcome

- New adaptations of existing assays for antimicrobial testing were generated for the highly-fastidious and slow growing bacteria of the genus *Bartonella*.

Obstacles Encountered Which Yet Remain Unanswered

- The synthesis of PNAs was unsuccessful accordingly, their ability to knockout expression of specific bacterial genes remains unanswered.
- The demonstration of successful translocation of Gfp-Bep fusion proteins was not definitively demonstrated as bacterial invasion can't be ruled out.

Intellectual Property Developed

U.S. Utility National Patent Application filed February 22, 2011: "Activity of New N-Acylated Ciprofloxacin Derivatives Against Facultative Intracellular Bacteria," Edward Turos, Ryan Cormier, Burt Anderson, John Thomas, Rebecca Kapolka, and Glenn Roma; Provisional Patent Application filed on February 19, 2010; "Bartonella as a Model Intracellular Pathogen for Developing Therapeutics," Disclosure filed on February 18, 2010 .

Poster Presentations

Bartonella; a Model Intracellular Pathogen for Developing Therapeutics. John C. Thomas, Rebecca J. Kapolka, Ryan Cormier, Edward Turos and Burt E. Anderson, *23rd Meeting of the American Society for Rickettsiology*, Hilton Head Island, SC, August 2009

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Burda, W., R. Cormier, L. Harrington, J. Edlinger, K.M. Kodigepalli, J. Thomas, R. Kapolka, G. Roma, B.E. Anderson, E. Turos, and L.N. Shaw. Antimicrobial properties of N-acylated ciprofloxacin derivatives. Submitted.

Introduction

The development of vaccines is widely considered to be one of the most important medical advancements of the 20th century. Current methods have been pushed to the limits of their potential. New techniques need to be developed and employed to combat a new generation of diseases and infections. There are several advantages to DNA vaccination. DNA vaccines are cost effective to produce, they can be easily stored, they are highly specific and their multivalent nature means that they could be combined to vaccinate against several different components simultaneously [1-3]. Either due to low expression or lack of immune recognition, injection of plasmid DNA alone does not elicit a strong enough immune response for protective vaccination. Electroporation (EP) is a non viral plasmid DNA delivery approach that effectively enhances plasmid expression [4, 5] and immunity [6-10].

EP requires the application of electric fields causing permeabilization of the cell membranes. The permeabilized membrane briefly contains "pores" that allow large molecules, like DNA, to enter the cell. Initial studies evaluating *in vivo* EP for transgene delivery and expression were performed on rat brain tumors [5] and rat livers [4]. Those studies demonstrated enhanced delivery and expression of plasmid DNA from EP mediated delivery. Successful EP mediated DNA delivery has been demonstrated in most tissue types and for several therapeutic and prophylactic indications such as cancer therapy, infectious diseases, wound healing, metabolic disorders and vaccines [11]. Recently several clinical trials have been initiated. Two clinical trials have been completed using EP, one assessing tolerability of intramuscular delivery [12, 13] and the other assessing toxicity and clinical utility of delivering pIL-12 intratumorally by EP to melanoma patients [14]. The latter demonstrated the safety, minimal toxicity, and feasibility for the use of EP in the clinic [14]. Since the successful completion of these studies, 19 others are currently active or recruiting. Five of those are involving DNA vaccination against infectious agents (clinicaltrials.gov; Keyword: Electroporation).

Initial *in vivo* EP DNA vaccine studies evaluated gene expression and immune stimulation from delivery of plasmids encoding either Hepatitis B Virus (HBV) protein or Human Immunodeficiency Virus (HIV) protein, gag, to the muscle. Their results confirmed that increased humoral responses to HBV [6] and cellular [9] immune response to HIV gag from EP compared to injection only (IO) of plasmid DNA. More recent studies have broadened the list of pathogens which EP has been successfully used *in vivo* to include other viral pathogens such as: Simian Immunodeficiency Virus [15-18], Severe Acute Respiratory Syndrome [19, 20], Influenza [21-25], West Nile and Japanese Encephalitis [26, 27], as well as Hepatitis B and C [28-32] and Human Papilloma Virus [33, 34]. EP delivered DNA vaccines expressing proteins of the parasitic infection *Plasmodium falciparum*, one of the parasites causing malaria [35], as well as bacterial infections like Bacillus *anthracis [36]*, *Clostridium botulinum [37]*, and *Mycobacterium tuberculosis* [38] have also been demonstrated to enhance immunogenicity. These results demonstrate the capacity of EP to enhance not only gene delivery and protein expression but also its ability to stimulate the host immune response against a wide variety of pathogens.

Current electrically mediated DNA vaccines employ painful invasive needle electrodes that are inserted into the muscle for electrical stimulation. The primary tissue used for *in vivo* EP is muscle because it is accessible, highly vascularized, multinucleated, and expresses DNA for long periods of time due to the post-mitotic nature of the tissue [39]. However, pain associated with administration is not desirable. As such, alternative delivery sites and methods have been explored. The skin is an attractive target for vaccination because of the high proportion of antigen presenting cells (APC) and large surface area. Recent studies, as well as work done in

our laboratory, demonstrated that intradermal electrically mediated DNA expression can be increased both locally and systemically [8, 40-44]. Electrodes developed for skin EP include: caliper, plate, tweezer, and clip electrodes as well as several needle electrodes [14, 45-48].

To develop an electrically mediated intradermal DNA vaccine we utilized the non-invasive multi-electrode array (MEA) for EP delivery. The MEA has 16 electrodes placed 2mm apart and is arranged in 4 rows [45]. Pulses are administered in a sequence that utilizes 4 electrodes at a time, forming 2 X 2 mm squares (9 total squares). Pulses are applied in pairs, in two directions, perpendicular to each other (18 pulses) for 4 rounds of pulsing (72 total pulses). Our lab previously demonstrated that this electrode, when used in a guinea pig skin model, could significantly increase reporter gene activity [45]. Conditions required for optimal expression were determined to be between 200-300 V/cm and 150ms.

Bacillus anthracis is a gram positive spore forming rod-shaped bacterium. The Centers for Disease Control and Prevention classify *B. anthracis* as a category A agent because "it can be easily disseminated or transmitted from person to person, causes a high mortality rate and public health impact, may cause panic and social disruption, and requires special action for public health preparedness." The current available vaccine is a recombinant protein vaccine delivered intramuscularly in a 5 regimen dose over the course of 18 months. Following this series, annual boosters are recommended [46]. The protein is isolated from a toxigenic non-encapsulated form of the bacterium V770-NPR1 [46-51]. Side effects have been noted in approximately one-third of vaccinated individuals including: injection site swelling, redness, and tenderness [52]. The primary component of this vaccine is a toxin associated protein called Protective Antigen (PA) [50]. This protein is involved in the formulation of both edema (ET) and lethal toxin (LT) in the bacterium [53] LT causes a disruption in the MAPKK pathway leading to cell death [54]. During infection PA is cleaved to its active 63KD form, which binds other active PA's forming a heptameric pre-pore. This PA complex binds edema and/or lethal factor and is translocated into the cell [53], where the toxins are secreted. Research demonstrates that some antibodies formed against PA can prevent toxin formation which is a critical component of vaccine development [55, 56]. For this reason most research conducted for the formulation of novel *B. anthracis* vaccines has utilized PA as a target. These PA vaccines have been shown to have varying success upon challenge [46-49, 51, 57-64]. Increasingly DNA has been used for vaccination. It has several advantages including: ease of production and storage, eliminating the need for the cold chain [65-67]. DNA vaccination has been shown to induce immune responses against several infectious agents. Original studies were conducted against HIV and HBV. Unfortunately, those responses were relatively low and did not sustain protective immunity. Methods of delivery were evaluated to determine whether these DNA vaccines could be enhanced. One of those methods was electroporation.

The primary goal of this study was to identify the appropriate delivery protocol for DNA vaccination with the MEA against *B. anthracis* PA and determine the potential for translation into a clinical setting. The most appropriate and well studied animal model for testing *B. anthracis* vaccines is the mouse. Therefore, this part of the study was designed to determine the prophylactic potential and appropriate conditions of a MEA mediated DNA vaccine against *B. anthracis* in a mouse model.

Specific Aim 2

Development of MEA mediated intradermal DNA vaccination against *B. anthracis*

a: Evaluate the effect of electroporotation with the MEA on the skin macroscopically and microscopically; assess damage and inflammation caused by Electroporation of the mouse tissue

b: Determine gene expression levels with Luciferase from MEA mediated intradermal injection

c: Evaluate the level of immune stimulation from DNA vaccination based on humoral immune responses with varying

1: DNA dose; 2: Treatment schedule; and 3: Electric Field

d: Evaluate the vaccine potential of the optimized delivery conditions against *B. anthracis* by *in vitro* toxin neutralization

Results

a. Effect of Electroporation with the MEA on the skin

Histology was performed to evaluate the skin tissue for damage at 175V/cm. IO samples show no gross visual difference in swelling or skin damage from EP treated animals. Histologically,

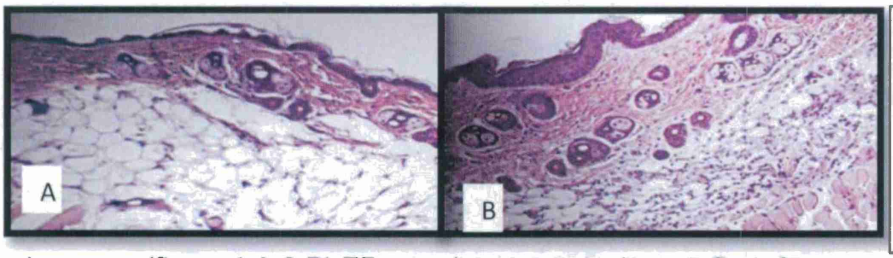

Fig 1. Effect of Electroporation on the mouse skin. Balb/c mice skin was treated with PA plasmid. Fig 4B was electroporated with 175V/cm immediately after treatment.

however, (figure 1 A & B) EP samples showed a large influx of cellular infiltrate as well as a thickening of the epidermis. No visual damage was seen to the skin.

b. Luciferase gene expression from intradermal electrically mediated delivery with the MEA in Balb/c mice.

In order to assess the ability of the MEA to enhance gene expression in a mouse model, previous experiments tested guinea pigs, Balb/c mice were injected with 50ul of GwizLuc (2mg/ml) intradermally on the left flank. Sites were electroporated with various electric fields with the MEA or 100V/cm with the 4PE. A control group of Injection of plasmid only was included (IO). Figure 2 shows that using the MEA luciferase expression can be increased and that the increase is field dependant. Higher electric fields result in

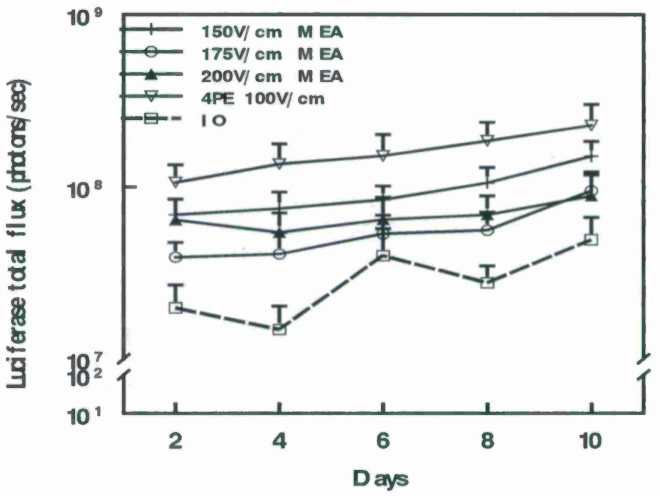

Fig 2. Luciferase expression in the mouse skin. Balb/c were treated as described in Methods with GwizLuc. Luciferin was injected i.p. and the mice were imaged over time in the Caliper Life Sciences IVIS Spectrum.

increased luciferase expression. However, all MEA conditions are greater than IO and demonstrate similar expression patterns as the control 4PE animals over time. Visual tissue damage was seen in animals treated with the MEA at 200V/cm. Though this condition represented the highest level of gene expression with the MEA conditions above 175V/cm will not be used to prevent damage.

c. Humoral immune response from intadermal electrically mediated delivery with the MEA against *B. anthracis* toxin protein PA.

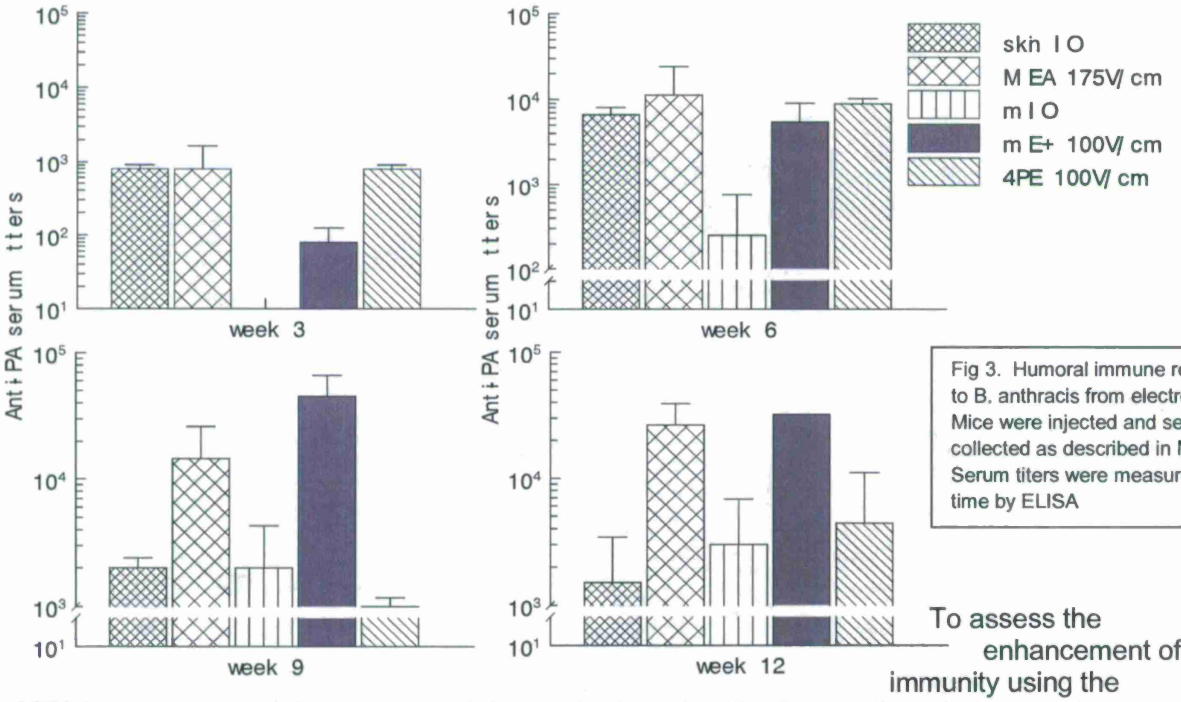

Fig 3. Humoral immune response to B. anthracis from electroporation. Mice were injected and serum collected as described in Methods. Serum titers were measured over time by ELISA

To assess the enhancement of immunity using the MEA in a mouse model we compared the production of antibodies to other electroporation devices. Mice were injected as described with pPA on the left flank for skin EP animals and in the gastrocnemius for muscle EP samples. Figure 3 shows that at early timepoints IO animals have higher expression than muscle injected groups demonstrating the benefit of using skin as the delivery location. However, over time those animals that received EP, regardless of delivery location, rapidly increased above IO groups. MEA EP samples increase steadily over time whereas muscle EP animals peak by week 9 and begin to drop off by week 12. Additionally, when comparing MEA skin delivered groups to 4PE skin delivered groups there is also a noticeable difference in antibody production. Despite the earlier noted increase in luciferase gene expression using the 4PE, MEA delivered groups produce approximately a five-fold increase in antibody production compared to the 4PE.

Optimization of delivery parameters for DNA vaccination with the MEA against *Bacillus anthracis*.

To determine the appropriate delivery conditions for intradermal DNA vaccination with the MEA against *B. anthracis* we evaluated three parameters. The first was the plasmid dose injected during each treatment (1). The second was the number of treatments each animal was given (2). We evaluated either a prime boost protocol (Day 0 and 14) or a prime plus two boost protocol (Day 0, 14 and 28). The final parameter was the electric field effect (3). We evaluated fields from 25 to 175V/cm. Our early gene expression studies demonstrated that fields greater

than 175V/cm caused visual tissue damage in the mouse model, so while 200V/cm represented the highest gene expression condition it was not included in further experimentation.

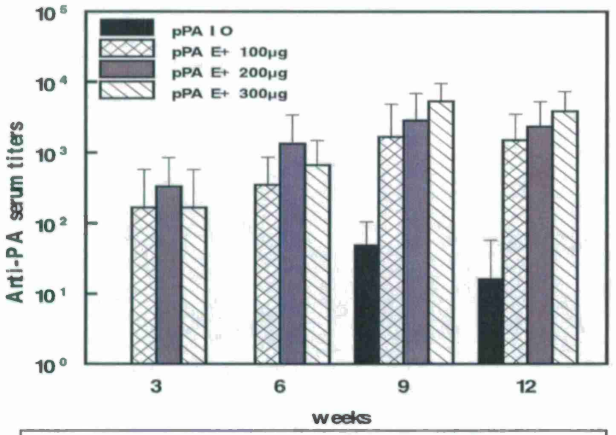

Fig 4. Effect of Plasmid Dose on MEA mediated Vaccination. Mice were injected with varying amounts of plasmid from 100 to 300µg. Serum collected as described in Methods. Serum titers were measured over time by ELISA

1. Plasmid Dose.

Differing amounts of plasmid were injected to compare its effect on immune induction. Plasmid was injected at 100µg, 200µg, or 300µg intradermally. EP was used for delivery at all three plasmid amounts. Serum was collected and antibody levels measured. Results, Figure 4, show very little differences in antibody production. All conditions are increased above IO at all time points. However, at early timepoints 200µg seems to be slightly increased over 100µg and 300µg. By week 9, 300µg had surpassed 200µg and maintained at week 12. These results do not generate any significant benefit to increasing plasmid dose. The largest differences affected by plasmid dose occurred at weeks 3-6. Over this time 200µg of plasmid shows the largest differences between groups for this reason we have selected to continue further experimentation with that dose.

2. Treatment number.

Animals were treated on either Day 0 and 14 or Day 0, 14, and 28. Antibodies were measured and plotted over time. The results, shown in Figure 5, demonstrate that at early time-points there may be some benefit to an additional treatment however, those animals given only one boost achieved antibody levels higher than the two boost group over time. This suggests that additional benefits from a third treatment would not be gained for prolonged immunity and would only be beneficial for short term immune responses.

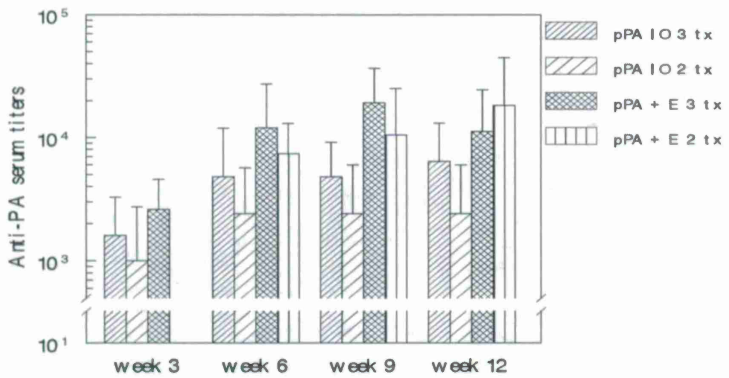

Fig 5. Effect Treatment number on MEA mediated Vaccination. Mice were injected and serum collected as described in Methods. Boosts were either given at Day 14 or Day 14 and 28. Serum titers were measured over time by ELISA

3. Electric field effect on antibody responses

As expected the electric field applied seems to have the most significant effect on antibody production. In Figure 6 we compared IO to MEA delivered groups with electric fields ranging from 25 to 175V/cm. Here, a distinct pattern of increased antibody production correlating with increasing electric field can be seen. At week 3, 125V/cm generates the highest response. However, by week 6, 175V/cm increases to about equal levels and remains statistically similar to 125V/cm. By week 9 significant increases of both 125 and 175V/cm are seen as compared to IO and 125V/cm is significantly greater than all other lower electric fields. At week 12 significant increases are again seen as compared to IO for both 125 and 175V/cm.

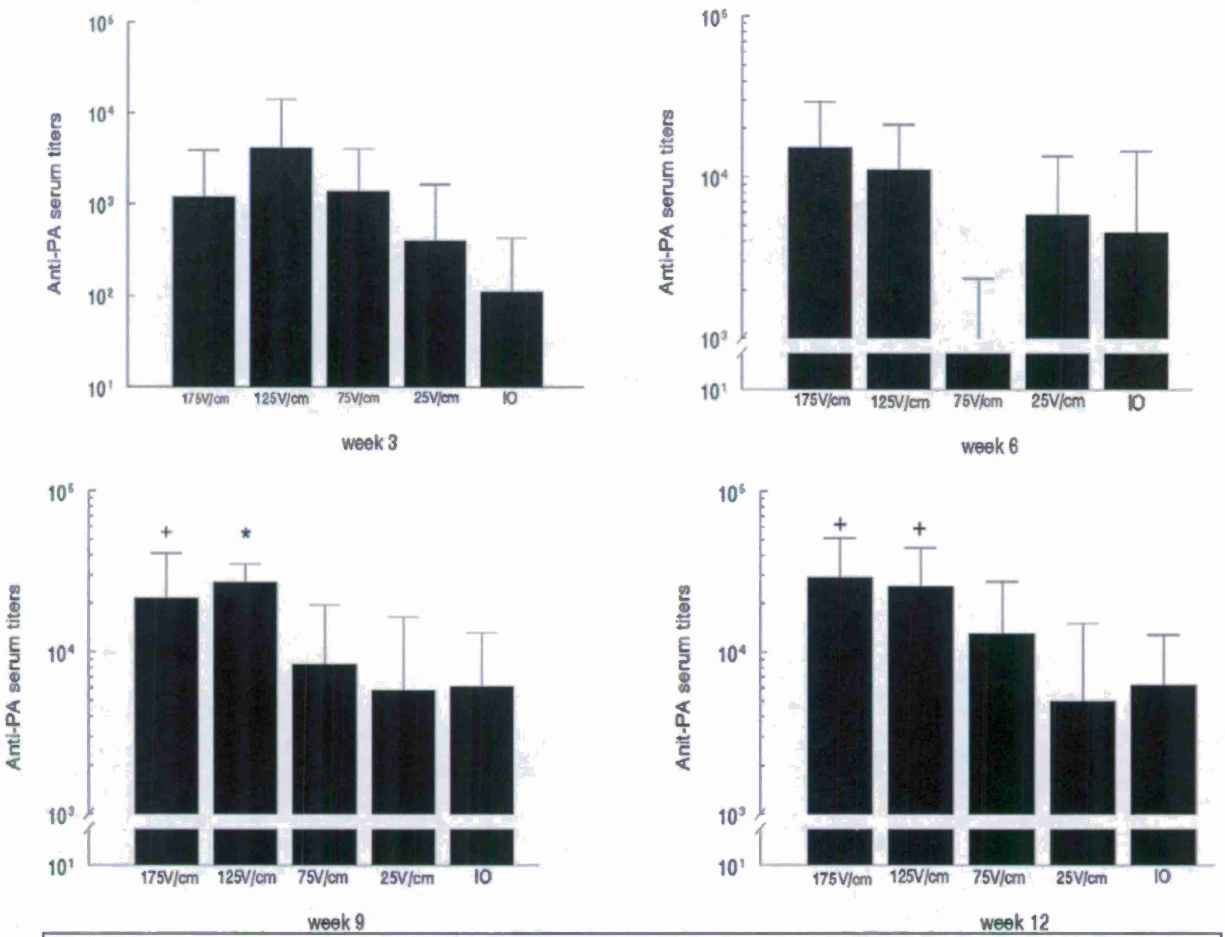

Fig 6 Effect of Electric field on MEA mediated Vaccination. . Mice were injected and serum collected as described in Methods. Electric field was varied from 25 to 175V/cm with constant pulse length of 150ms. Serum titers were measured over time by ELISA. *=significance over all lesser electric fields and IO (p of 0.05) and += significance over IO (p of 0.05).

d. Vaccine potential of the MEA against *B. anthracis*

The critical question is whether a protective immune response can be generated using this delivery method. To assess this, a toxin neutralization assay was performed to determine the titer of neutralizing antibodies generated from our "optimized" delivery conditions. Table 1 shows that 3 out of 5 mice could generate neutralizing antibodies using the MEA at 175V/cm and 2 out of 5 for 125V/cm. IO and EP only groups did not have any neutralizing activity.

Condition	Peak serum titer	Serum Dilution	# of mice with TNA's
pPA Injection only	3,200	50	0 of 5
Backbone + EP 175V/cm	0	50	0 of 5
pPA + EP 175V/cm	12,800	50	3 of 5
pPA + EP 125V/cm	25,600	50	2 of 5

Methods

Animals and injections: 6-8 week old female balb/c mice were intradermally injected at two sites on the left flank with 50µl of plasmid for experimental animals. Experimental mice were boosted either once or twice 14 days after the previous treatment (Day 14, Day 28). All experiments included control animals of 10µg muscle injected recombinant protein as well as injection only. Recombinant protein injections were administered at Day 0, 14, and 28. Mice were bled by tail vein at various time-points. All animals were anesthetized with 2-3% isoflurane + O_2 for treatments.

Plasmids: The plasmids used for these experiments were pSecTagPA and pCMVER/PA at various concentrations for B. anthracis studies. The PA plasmids were generously donated by the Hahn lab (Germany). Reporter assays were done using pGwizLuc (Aldevron) at 2mg/ml.

Electroporation: The multi-electrode array was used at applied electric fields ranging from 25 to 225V/cm in mice but always maintained constant pulse duration and delay of 150ms. A sequence of 9 4X4 squares was applied 4 times for a total of 72 pulses. Electrodes were circular, gold plated and flat at the end with a 0.2mm diameter.

In vivo Bioluminescent Imaging: The Caliper life sciences IVIS Spectrum was used for live animal bioluminescent imaging. Animals were injected i.p with 15mg/ml luciferin. 20 minutes post luciferin injection the animals were imaged and relative light units measured. All luciferase data is represented as average total flux (photons/sec) per injection site.

Histology: Skin samples were taken from both mice for histological analysis. Mouse skin was collected 48 hours after treatment and fixed in formalin. Hematoxylin & Eosin staining was performed to assess inflammation and damage.

Serum collection: Mice were bled at various timepoints by tail vein. Blood was collected and serum isolated in serum separator tubes. Serum was diluted two fold starting at 1:1000.

Indirect ELISA for the determination of PA specific antibodies: Briefly antigen was coated at 0.1 to 1µg/well and incubated overnight at 4°C. Plates were blocked with 5% skim milk buffer (anti-PA) for 2 hours at 37°C. Samples were diluted in blocking buffer and incubated for 1 hour at 37°C. HRP conjugated Secondary antibodies (Santa Cruz) were diluted in blocking buffer to working concentration and added for 30 to 60 minutes in dark. R&D substrate was added for 10 minutes and stopped with 2N H_2SO_4. Plates were read at 450nm and results represented as mean titers.

Toxin neutralization assay: J774A.1 murine macrophages were cultured in DMEM media supplemented with 5% FBS, 10mM HEPES buffer, and Pen-Strep. 50,000 cells/well were plated in 96 well cell culture plates. The next day serum was diluted starting at 1:50 in media and incubated for one hour with 100-200ng/ml protective antigen. Lethal Factor was added to the Serum/Protective antigen mix at a final concentration of 80-160ng/ml. Media was removed from the cells and the serum/PA/LF mix was added to the macrophages for 4 hours at 37 and 5% CO2. All plates contained a titration curve to confirm that the concentration of toxin used was sufficient to cause 95% cell death. Following the 4 hour incubation an MTT assay was performed to determine cell viability.

Discussion

These data demonstrate that the MEA can be effective for the use in electrically mediated DNA vaccination and it has potential for use as an EP delivery device for intradermal DNA vaccination against *B. anthracis*.

DNA vaccination is advantageous because it does not integrate into the host DNA, it is cost effective to produce and easily stored, it can be highly specific for tissue and/or cell type and can be made to vaccinate against multiple agents simultaneously. The skin is an ideal target for DNA vaccination due to the large surface area and presence of antigen presenting cells like langerhan's and dermal dendritic cells, specialized for induction of immunity [69].

However, injection of plasmid alone does not induce high enough immune responses to be protective. EP is one method that has been shown to increase both plasmid expression as well as immunity. Previous EP methods have involved painful penetrating electrodes that go into the muscle to facilitate delivery. Further advancements have been made using non-penetrating electrodes such as caliper and plate electrodes. However, these electrodes require high voltages to enhance delivery and therefore can cause tissue damage. In this study, we have evaluated a non-penetrating electrode which reduces the gap width between electrodes to 2mm thereby reducing the absolute voltage applied and preventing visible tissue damage while still increasing plasmid expression and immunity.

As expected from our previous publication [45], EP with the MEA enhanced expression. While the exact reason for the effectiveness of EP remains unknown, increased plasmid expression at least in the case of DNA vaccination, plays an important role in recognition by the immune system [70]. EP has been shown to have an adjuvant effect by recruiting immune cells to the site of pulse application [71]. In our study, we saw an influx of nucleated cells from EP treated samples. These cells are most likely neutrophils and macrophages based on morphology. This is most likely a combination of both an EP mediated adjuvant effect and increased plasmid expression. The induction of macrophages and polymorpho-nucleated neutrophils is indicative of a chronic inflammatory response. While the perception of prolonged inflammation is typically negative in our case it indicates that the expression of the plasmid is present for a prolonged period of time, giving the immune response enough time to perform its function. Based on our earlier work [45] we would expect this prolonged expression to decrease after approximately 14 days, therefore allowing the body to heal and not generate deleterious effects from inflammation.

These findings seem to correlate with our antibody data, where an increase in the presence of specific antibodies was measured over time. These antibodies were significantly increased as compared to injection only. The enhanced intensity of humoral immunity by EP with the MEA corresponds to previously published skin EP results [72-75]. The most recent comparable publication evaluated a minimally invasive device for protective vaccination against influenza

[76]. While their results were only presented as neutralizing titers against flu and cannot be compared directly we believe that our electrode design generates immune responses of equal quality without tissue penetration.

We determined that the MEA can induce protective antibodies with only a prime boost protocol and just 200ug of plasmid per treatment in a mouse model against *B. anthracis* . This is substantially less than the current vaccine protocol for *B. anthracis* which requires 5 initial treatments and annual boosters [77]. Visual tissue damage was minimized by appropriate EP parameter of less than 200V/cm without sacrificing immune responses. In fact though higher EP fields may result in greater plasmid expression as demonstrated in figure 2 this does not correlate with antibody responses which were significantly increased with EP fields as low as 125V/cm. Additionally toxin neutralization was achieved in 60% (3/5) of mice with 175V/cm and 40% (2/5) with 125V/cm. We saw no significant increases in immune response from increasing either the dose (fig 4) or the number of treatments (fig 5). A dose of 200µg and 2 treatments is considered the minimally sufficient dose for vaccination. Additionally histological analysis (fdata not shown) shows an influx of cellular infiltrate to the treatment site as early as 48 hours after the initial treatment. This is not seen in the IO. Increases in cellular infiltrate may represent protective effects that are not represented by measurement of antibody responses and neutralizing activity. Early work conducted with the gene gun was also suggestive of this effect [78] In that study toxin neutralization was quite low and in several cases non-existent however several of these animals were protected in lethal challenge assay. Future work on the development of this vaccine would need to include these types of challenge experiments to confirm these effects.

Additionally when conducting our neutralizing assays, those animals that did not elicit 50% protection, and were therefore not considered protected, did still demonstrate some protective effects at 20-40%. This was not seen in the IO or backbone controls where at least 95% cell killing occurred. Despite not being increasing cell viability enough to be considered protected, there was some response in every MEA treated animal at 175 and 125V/cm.

When comparing our results to other published anthrax vaccines. The total IgG responses obtained in this study exceed most other DNA vaccines tested in Balb/c models [79-84]. One recent publication demonstrating substantially higher total IgG in a Balb/c model required additional boosters or addition of recombinant protein boosts [85] or the use of signaling adjuvants. Some reports with DNA vaccination in other mouse models are slightly increased over our results [86, 78, 87-89] and this may be explained immunogenicity of the models. Balb/c mice are not highly susceptible to challenge from non-encapsulated toxigenic strains of *B. anthracis*, whereas mouse models like A/J mice are highly susceptible [90]. In one comparison, of A/J and Balb/c mice and the development of humoral immunity against *B. anthracis*, the A/J mice developed almost a ten-fold higher response than the Balb/c mice [84].

These results compare favorably to muscle EP as well. Two other studies, one in mice [86] and one in non-human primates [57], have been conducted specifically evaluating the use of EP to deliver a *B. anthracis* vaccine. Our results are similar to the mouse study demonstrating approximately 25000 titers and peaking at similar time points between 6-9 weeks. While our study required additional DNA the use of the non-invasive MEA provides a positive advancement to this study. The NHP experiment was conducted with penetrating needle electrodes into the muscle. Their results showed the development of protective immune response [57]. While it would be difficult to compare our results to these, we feel that their data corroborate the claim that EP could be an effective delivery method for DNA vaccination against *B. anthracis*.

The data represented here demonstrate the capability of the MEA to increase plasmid expression, immune cell infiltrate and inflammatory response. This information presents a potential new method for DNA vaccination that may be translatable to humans. Most importantly we were able to generate protective immune responses against potential bioterror weapon *B. anthracis*. We believe our method may be a way of making this treatment even more tolerable and perhaps reducing not only the invasiveness of the procedure but also reducing the dose.

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June 28, 2012

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To Whom It May Concern:

Enclosed is the written Final Technical Report (two copies for distribution per the award notice) for Dr. Burt Anderson (University of South Florida) DARPA Award # HR0011-08-0087, Drug Discovery, Design, and Delivery.

If you have any questions, please feel free to call me at (813) 974-1914 or send an e-mail message to nina2@usf.edu.

Sincerely,

Nina R. Pemberton

Nina R. Pemberton
Administrative Specialist

/nrp

Enclosures

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